EFFECT OF RADIATION PROCESSING ON LIPID METABOLISM IN SOME INDIAN VEGETABLES: IMPACT ON AROMA QUALITY

By

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Dedicated to My Parents...

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SYNOPSIS

Vegetables are essential components of a healthy diet and provide essential nutrients and bioactive phytochemicals. They represent no specific botanical group and exhibit a wide variety of plant structures. Vegetables of *Brassicaceae* family are one of the most popular vegetables consumed all over the world and considered to be a good source of bioactive phytochemicals. Additionally, these vegetables are increasingly becoming a research model in plant science, due to importance of their bioactive metabolites. Adequate consumption of these vegetables has been associated with a reduced risk of chronic disease like cancer [1].

Brassica vegetables are widely marketed as a minimally processed (MP) form due to the associated convenience. Apart from maintaining microbial safety, maintenance of fresh like

characteristics is the main criteria determining consumer acceptability of the product. Various post harvest processing techniques are applied for processing for fresh products [2].

Food irradiation is a physical means of food processing involving exposure of food products to gamma rays or electron beam for eliminating disease-causing microorganisms. The potential application of ionizing radiation in food processing is based mainly on the fact that ionizing radiations damage very effectively the DNA thereby inactivating living cells including microorganisms [3]. The process has recently been recommended for microbial decontamination of fresh vegetables of the Brassica species such as lettuce [4]. This has lead to a greater interest in the use of radiation processing particularly for vegetables of Brassica family.

Plants when exposed to stress, adapt to unfavorable conditions through genetically determined stress resistance. Unlike typically processed foods, fresh-cut products consist of living tissues and post harvest processing treatments including radiation processing can act as stress bringing about change in post harvest physiology of the product. There are few reports on the impact of radiation processing on the post harvest physiology of vegetables particularly that of Brassica species. Consumers and researchers are, therefore, worried about the nutritional and chemical quality of such radiation treated produce.

Membranes are the main targets of degradative processes induced by stress. ROS produced during water radiolysis tend to bring about impairment in membrane integrity. Effect of radiation processing on lipids mainly phospholipids and the consequent formation of off-odors in high fat containing foods have been extensively reported in literature. However, effect of radiation processing on the lipid profile of Brassica vegetables is yet to be explored.

Polar glyceroglycolipids are the major membrane lipids in photosynthetic organisms such as higher plants and algae. Monogalactosyldiacyl glycerol (MGDG), digalactosyldiacylglycerol

(DGDG) and sulfoquinvosyldiacylglycerol (SQDG) account for 90% of the total lipids in the chloroplast thylakoid membrane. Unsaturated fatty acids liberated from these lipid species have been demonstrated to be the precursors in the formation of the green aroma compounds via lipoxygenase (LOX) pathway [5]. Vegetables are generally characterized by their green odors. Volatile aliphatic C6 compounds involving C6 aldehydes and alcohols and their corresponding hexyl esters, collectively known as green leafy volatiles (GLVs), decisively contribute to these characteristic green odors[5]. A group of lipid hydrolyzing enzyme called lipases release fatty acids from the membrane lipids which are acted upon by enzymes such as LOX and further by hydroperoxide lyase (HPL) of the LOX pathway to form the GLVs (Hatanaka, 1996). Various abiotic stresses such as salt stress, draught stress and mechanical stress are known to bring about changes in GLV content [5]. However, impact of radiation stress on the green odors of leafy green vegetables and consequently on their aroma quality has not been investigated so far.

Isothiocyanates are the major group of compounds known to impart characteristic aroma and taste to Brassica vegetables [6]. Isothiocyanates are the hydrolytic products of glucosinolates (GSL), bioactive compounds present in Brassica vegetables. These compounds have been reported to be mainly responsible for the observed chemoprotective activity of these vegetables. Various stresses are known to affect the GSL profile of Brassica vegetables. Selective induction of GSL brought about by jasmonates has been widely demonstrated [1]. These compounds are also byproduct of lipid metabolism. No studies so far have dealt with effect of radiation stress on these compounds. Since any change in the GSL content may affect the aroma, taste as well as nutraceutical quality of Brassica vegetables assessing the effect of radiation processing on these compounds is important to determine the overall quality of the product.

There are few reports on the impact of radiation processing on the post harvest physiology of vegetables of Brassica species. A correlation between physiology, biochemistry and alteration in

sensory and nutritional parameters by postharvest treatments is needed. Molecular knowledge of radiation stress response and tolerance mechanism can provide insight into how these are regulated and could be the basis for increasing nutritional and sensory quality of these products. The present thesis aims at understanding the effect of gamma irradiation on post harvest physiology of Brassica vegetables with respect to aroma profile and further understanding the mechanism of physiological changes at biochemical and molecular level.

Chapter 1 of the thesis introduces the subject of food irradiation with special emphasize on irradiation of vegetables of Brassicaceae family and describes the scientific literature related to the present work. Based on the review of available literature, it was found that considerable work has been done on the effect of different abiotic stresses including UV radiation on vegetables and fruits. However, few reports have dealt with the impact of radiation processing on the post harvest physiology of brassica vegetables. The present thesis deals with the effect of gamma irradiation on lipid metabolism with respect to aroma quality in brassica vegetables and further understanding the mechanism of changes at biochemical and molecular level in cabbage (*Brassica oleracea var capitata).*

Chapter 2 of the thesis describes the materials and experimental methods. Vegetable samples of different varieties were obtained from Dr. Panjabrao Deshmukh Agricultural University, Maharashtra and from local markets in Mumbai. Irradiation was carried out using a cobalt-60 irradiator (GC 5000, Board of Radiation and Isotope Technology, India) at BARC, Mumbai.

Nikersons-Likens simulataneous distillation extraction apparatus and solid phase microextraction technique was used for isolation of aroma compounds. Further analysis was done by GC/MS. The lipid constituents were studied using TLC and GC/MS. The non volatile

constituents that included GSL, lipids and phenolics were studied by TLC, HPLC and LC/MS analysis.

Activities of different enzymes were assayed by reported spectrophotometric methods. Expression of associated genes was studied by qRT PCR analysis. RNA extraction was done using TRI reagent. cDNA preparation was done using SuperMix for qRT-PCR (Invitrogen, CA, USA).

Gamma irradiation was used for shelf life extension of MP shredded vegetables. Sensory quality was assessed by a sensory panel through hedonic testing. Browning was evaluated by colorimeter and texture through texture analyzer according to the standard protocols. Nutritional parameters like vitamin content, total phenolic content and antioxidant properties were studied according to standard AOAC protocols.

Chapter 3 deals with the results obtained. It has been divided into following subsections.

3.1 Screening of vegetables: Preliminary screening of vegetables was done based on their aroma profile. Effect of radiation processing (0.5–2 kGy) and storage on volatile oil constituents of 4 vegetables, namely cabbage, cauliflower, radish and broccoli were studied. Among the volatiles green leafy odor is contributed mainly by *trans*-hex-2-enal and *cis*-hex-3-enol that were prominent in cabbage, broccoli and cauliflower, while these compounds were not detected in radish. A 78% increase in GLV content was noted as a result of irradiation (2 kGy) in cabbage while their concentration increased by 15% in broccoli. No change in GLV content was found in cauliflower. Allyl isothiocyanate (AITC), the major aroma compound identified in cabbage and responsible for its characteristic odor, was found to get enhanced by 80% in cabbage. The aroma quality of each of these vegetables was further analyzed by a sensory panel. An enhancement in characteristic aroma was perceived by the panelist in cabbage. No change in sensory profile

with respect to aroma as a result of gamma irradiation was noted in other samples. Since gamma irradiation was found to affect aroma quality of cabbage which in turn directly affected its sensory quality this vegetable was chosen for further studies.

3.2 Mechanism of irradiation induced GLV enhancement: The mechanism of enhancement in GLVs (*trans*-hex-2-enal) induced by gamma irradiation is discussed further. *trans*-2-Hexenal, is derived from the LOX pathway via linolenic acid, hence the nature of the lipids and their fatty acid composition was investigated. MGDG and TAG were the major lipid species identified in cabbage. A radiation dose dependent decrease in these lipid species with an increase in free fatty acids was noted. Among the individual fatty acids, a dose dependent decrease in linolenic acid was clearly observed. As LOX, HPL and lipases are the main enzymes of LOX pathway, it was of interest to understand the role of these enzymes in enhancing the content of *trans*-hex-2-enal during radiation processing. No enhancement in the activity of any of these enzymes was noted at different radiation doses and at different time points. Hence, lipid radiolysis and a consequent increased availability of free linolenic acid for the enzymes of LOX pathway resulted in enhanced *trans*-hex-2-enal in the radiation processed cabbage in the present study.

3.3 Mechanism of irradiation induced AITC enhancement: Gamma irradiation induced enhancement in AITC content was studied further. AITC is known to be derived from sinigrin, the predominant GSL of cabbage, effect of radiation processing on this compound was studied. An increase in the content of sinigrin was noted immediately after irradiation. A linear increase was observed from 0.5 to 1kGy that remained constant beyond a dose of 1kGy. A good correlation was also noted between increase in AITC and sinigrin content. Thus the increased AITC observed in the steam distilled volatile oils from radiation processed vegetable could be the result of hydrolytic breakdown of more available sinigrin in the treated samples. Different postharvest stress type treatments like UV-light, exogenous application of jasmonates etc has

been shown to cause an accumulation of secondary metabolites like GSLs in vegetables. To the best of our knowledge this is the first report on gamma radiation induced enhancement in GSL in any vegetable.

A number of environmental conditions can enhance GSL content significantly. Selective induction of GSL by jasmonates and GLVs has been widely reported. These compounds are signalling molecules in plants and produced immediately in response to stress. Interestingly, these molecules are byproducts of LOX pathway and are derived from linolenic acid. Hence a correlation between linolenic acid enhancement during radiation processing and jasmonate content was speculated. However, no change in the content of these signalling molecules was noted in samples subjected to different radiation doses at different time point thus ruling out any role of these compounds in GSL enhancement in the present study. Effect of GLV on GSL content was also studied by storing the sample in atmosphere of GLVs. No change in GSL content was seen due to GLV treatment hence thus ruling out any role of GLVs in enhancing GSL during irradiation.

Mechanism of radiation induced GSL enhancment was further investigated at molecular level. Transcriptional analysis of genes associated with GSL biosynthesis revealed an alteration in their expression on exposure to gamma irradiation. *MYB28, CYP79F1, CYP83A1* and *SUR1* were found to be up-regulated resulting in an enhancement in content of aliphatic GSL. Hence increase in GLS content was accounted solely to be the effect of gamma irradiation without intervention of any other signalling molecule. To the best of our knowledge this is the first report on effect of gamma irradiation on GSL biosynthesis genes.

3.4 Application of gamma irradiation to develop MP shredded cabbage: Gamma irradiation (0.5-2.5 kGy) in combination with low temperature (4-15 °C) storage was attempted to increase

shelf life of cabbage wrapped in cling films. A maximum extension in shelf life of 8 days, while retaining its sensory and microbial quality, was achieved when the product was irradiated to 2 kGy (10 °C). Gamma irradiation also inhibited browning at cut edges resulting in improved visual appeal. An increase in antioxidant activity was observed with respect to $DPPH^{\cdot}$ and $OH^{\cdot}$ scavenging ability while the ferric reducing property and $NO^{\cdot}$ scavenging activity remained unaffected. Total phenolic, flavonoid and vitamin C content remained unchanged due to irradiation. Gamma-radiation induced inhibition of browning in MP shredded cabbage stored (10°C) up to 8 days was further investigated. In the control samples, phenylalanine ammonia lyase (PAL) activity increased during storage that could be linearly correlated with enhanced quinone formation and browning. No significant change was observed in polyphenol oxidase, peroxidase and in the total as well as individual phenolic content in both control and irradiated samples. Transcriptional analysis showed an up-regulation in PAL gene expression in the control samples during storage. Gamma irradiation (2kGy) resulted in inhibition of browning as a result of down-regulation in PAL gene expression and a consequent decrease in PAL activity. The present work is the first report on the mechanism of gamma irradiation induced browning inhibition in vegetables.

AITC is known to inhibit browning in cut vegetables whose content gets enhanced due to irradiation. In order to elucidate the role of AITC in radiation induced browning inhibition cabbage samples were stored in AITC saturated atmosphere. AITC was found to effectively increase shelf life of stored MP cabbage at 10 ºC by 5 days. Transcriptional analysis of PAL gene expression showed an initial up-regulation (0.8 fold) followed by shift to the basal value thus decreasing PAL activity in AITC treated samples. However, the decrease was less compared to the irradiated sample thus suggesting no role of AITC in radiation induced browning inhibition.

Chapter 4 is the concluding chapter of the thesis. This chapter discusses the possible future directions that can be explored further.

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CHAPTER 1

INTRODUCTION

Preamble

Food is one of the basic necessities of life. Eating is a natural phenomenon of ingestion, digestion, absorption and utilization of food for sustenance of life. Food provides energy and nourishment, and all foods come directly or indirectly from plants, of which considerable amount are vegetables.

1.1 Vegetables and their importance

Vegetables are essential components of a healthy diet. They represent no specific botanical group and are defined as fresh and edible plant or plant parts, that are typically either leaf, stem, or root and can be eaten either in the raw or cooked form. Epidemiological studies have provided evidence that adequate consumption of vegetables has been associated with a reduced risk of chronic disease like cancer, cardiovascular diseases, diabetes, Alzheimer disease, cataract, and age-related functional decline [1]. Table 1 lists the proximate composition of selected vegetables. The nutritional value of vegetables lies in their micronutrient content, fiber content and bioactive phytochemicals [2].

Micronutrients in vegetables generally comprise of vitamins and minerals which are required by humans in small quantities to orchestrate a range of physiological functions. Vegetables are rich source of vitamins like vitamin A, vitamin C, several B vitamins, vitamin E and vitamin K. They are also abundant in major minerals such as Na, K, Ca, Fe, Zn and Se [3,4]. Although these micronutrients are required in trace amounts but their deficiency causes a number of diseases in human.

Vegetables are also a good source of fibre that are mainly constituted of non-starch polysaccharides (cellulose, hemi-cellulose, pectins, gums and mucilages), lignin and

oligosaccharides in varying proportions and combinations. Health benefits of fibres include their water retention capacity that aid in improving bowel movements, glucose tolerance, lowered total LDL blood cholesterol levels and prevention of some cancers mainly that of the colon. Green leafy vegetables also have high quantities of cellulose necessary for digestion and in preventing constipation.

Vegetables also possess bioactive phytochemicals with proven health benefits (Table 2). Phytochemicals are the compounds found in vegetables which provide health benefits for humans beyond those attributed to macronutrients and micronutrients. They protect plants from disease, damage and contribute to the plant's color, aroma and flavor. More than 4,000 phytochemicals have been cataloged [5] (American Cancer Society 2002) and are classified by protective function, physical and chemical characteristics [6]. Three major classes are the terpenes (including the subclasses carotenoids and limonoids); phenols (including the subclasses flavonoids and isoflavones); and the sulfur containing compounds (including the subclasses the glucosinolates and dithiolthiones) [6]. Broccoli, cabbage, carrots, onions, garlic, tomatoes, beans and legumes are common vegetable sources.

The phytochemicals, either alone and/or in combination, have tremendous therapeutic potential in curing various ailments [7]. These are often considered as nutraceuticals and occur at the intersection of food and pharmaceutical industries. The term nutraceuticals, coined by Dr. Stephen de Felice, is derived from the words "nutrition" and "pharmaceutical", is a food or food product that provides health and medical benefits, including the prevention and treatment of diseases [8]. The plant nutraceuticals are formed through metabolism and are interconnected through different biosynthetic pathways that are affected by various external stimuli. Certain

internal or external factors play an important role in contributing to the metabolite profile of vegetables leading to an alteration in phytochemical content, thus changing the nutritional value for human beings. The USDA dietary guidelines recommend consuming 3 to 5 servings of vegetables daily [9]. WHO recommends a daily intake of 400 grams of fruit and vegetables [10]. However, in most cases fruit and vegetable intake is much below the recommended dietary guideline. The main barriers to achieving sufficient fruit and vegetable intake identified by consumers is the lack of sufficient time, difficulty in preparation and lack of attractiveness for including them in the daily diet. This has stimulated the need to design strategies for enhancement of nutraceuticals in crop tissues, such as the proposed classical breeding [11], genetic engineering [12] or use of elicitors for enhancement of the specific secondary metabolite production.

Table 1 Proximate composition of selected vegetables

	Bottle Gourd	**Cabbage**	**Cauliflower**	**Carrot**	**Potato**	**Radish**	**Spinach**
Protein (g/100g)	*1.2±0.06*	*1.6±0.2*	*1.8±0.06*	*1.5±0.01*	*1.9±0.1*	*1.3±0.04*	*2.1±0.15*
Fats (g/100g)	*0.2±0.02*	*0.2±0.01*	*0.2±0.02*	*0.2±0.00*	*0.2±0.01*	*0.1±0.01*	*0.38±0.01*
Carbohydrates (g/100g)	*3.75±0.03*	*4.8±0.01*	*4.8±0.01*	*10.4±0.21*	*19±0.15*	*4.56±0.04*	*4±0.12*
Fiber (g/100g)	*0.7±0.01*	*0.9±0.3*	*0.8±0.06*	*0.6±0.01*	*0.4±0.05*	*0.9±0.01*	*0.6±0.01*
Energy (g/100g)	*15±0.12*	*24±0.12*	*25±1.0*	*40±0.5*	*81±0.29*	*23±0.12*	*27±0.29*
Vitamin B_1 (mg/ 100g)	*0.03±0.01*	*0.06±0.01*	*0.09±0.04*	*0.05±0.02*	*0.1±0.06*	-	*0.13±0.01*
Vitamin B_2 (mg/ 100g)	*0.05±0.08*	*0.05±0.02*	*0.08±0.02*	*0.05±0.01*	*0.05±0.04*	-	*0.15±0.03*
Vitamin C (mg/ 100g)	*12±0.07*	*55±0.06*	*45±0.09*	*15±0.08*	*12±0.05*	-	*76±0.08*
Ca(mg/ 100g)	*12±0.03*	*52±0.03*	*23±0.12*	*39±0.06*	*8±0.02*	*33±0.03*	*76±0.06*
P(mg/ 100g)	*37±0.01*	*44±0.5*	*44±0.01*	*26±0.05*	*46±0.01*	*28±0.02*	*84±0.15*
Na(mg/ 100g)	*1.7±0.01*	*8±0.09*	*53±0.03*	*32±0.01*	*11.3±0.06*	*63.9±0.06*	*58.5±0.03*
K(mg/ 100g)	*87±0.02*	*12±0.04*	*135±0.18*	*102±0.12*	*240±0.09*	*10±0.07*	*202±0.19*
Fe(mg/ 100g)	*0.8±0.01*	*0.51±0.0*	*0.82±0.02*	*1.4±0.06*	*0.75±0.09*	*0.7±0.08*	*6±0.07*

Modified after Karrie Henman 'Nutrition & infosheet for health professionals

Table 2 Phytochemicals in vegetables

CLASS	PHYTOCHEMICAL	FUNCTION	SOURCE
TERPENOIDS			
Carotenoids	Beta Carotene	Antioxidant and precursor to vitamin A	Carrot, Sweet potato
	Lutein/Zeaxanthin	Macular pigment, protects the eye from macular degeneration	Spinach, Kale , Corn
	Lycopene	Anticancerous	Tomato, red pepper
Saponins		Aid in reducing blood cholesterol	Beans, Legumes
POLYPHENOLS			
Flavonoids	Quercetin	Antioxidant, anticancerous, effective against asthma	Broccoli, cabbage, onion
	Catechin	Antioxidant, anticancerous, may reduce the risk of heart disease	
	Isoflavones	Lower blood pressure and cholesterol	Soybeans
	Naringin	Antioxidant	
Phenolic acids	Ellagic acid	Anticancerous	
	Gallic acid	Antioxidant	
GLUCOSINOLATES			
	Isothiocyanates,	Anticancerous, antioxidant	Cabbage, broccoli, cauliflower, kale
	Indoles	Anticancerous	Cabbage, broccoli, cauliflower, kale

1.2 Minimal processing of vegetables

Minimally processed products are a growing segment in food retail establishments owing to the associated convenience with minimum preparation time before consumption (Table 3)[13]. These products are ready-to-eat (RTE) fresh fruits and ready-to-cook (RTC) vegetables that have been processed to increase their convenience without significantly altering their fresh-like characteristics [14]. In Europe the market for minimally processed fruits and vegetables is on the rise since 1990s. Similar scenario exists in USA where packaged salads are the second-fastest selling item in grocery stores followed by fresh-cut vegetables [15]. In India a major share of all produce sold in the retail market is known to be accountable to fresh-cut produce [16].

The IFPA defined fresh-cut produce as: "*trimmed, peeled, washed, and cut into 100% usable product that is subsequently bagged or prepackaged to offer consumers high nutrition, convenience, and value while still maintaining freshness*" [17]

The USDA and FDA defined "fresh" and "minimally- processed" fruits and vegetables as: *fresh-cut (pre-cut) products which have been freshly-cut, washed, packaged and maintained with refrigeration. Fresh-cut products are in a raw state and even though processed (physically altered from the original form), they remain in a fresh state, ready to eat or cook, without freezing, thermal processing, or treatments with additives or preservatives*[18]. The various steps involved in minimal processing of vegetables are shown in Fig 1. Processing operations and subsequent storage tends to deteriorate the quality of these products. Maintenance of overall sensory and nutritional quality as well as microbial safety of fresh cut produce remains a major challenge for production of minimally processed food.

Table 3 Commonly available minimally processed fruits and vegetable

Food item	Minimally processed form
Vegetables	peeled and sliced potatoes
	shredded lettuce and cabbage
	washed and trimmed spinach
	carrot and celery sticks
	cauliflower and broccoli florets
	packaged mixed salads
	cleaned and diced onions
Fruits	
	chilled peach, mango, melon and other fruit slices
	peeled and cored pineapple
	fresh sauces
	peeled citrus fruits

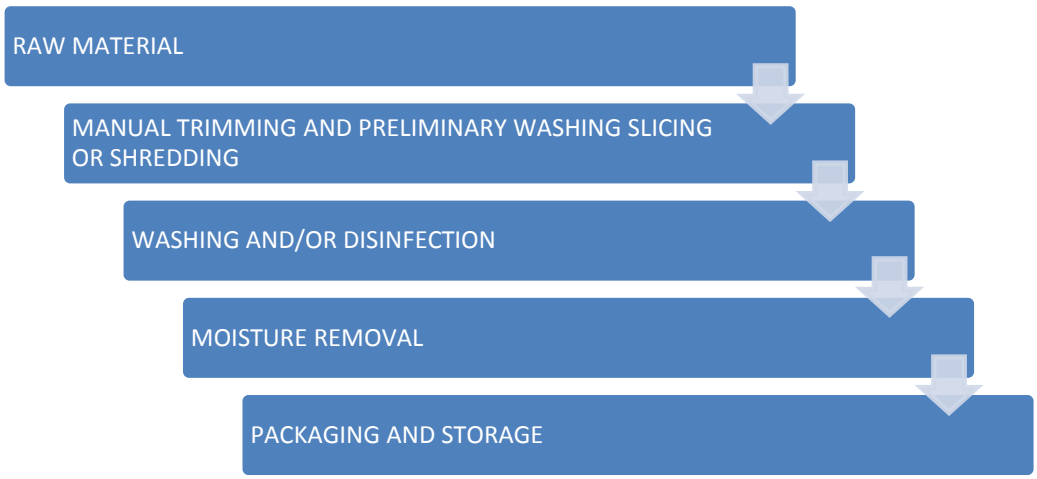


Fig 1 Flow diagram for the production of minimally processed vegetables

1.2.1 Factors contributing to the quality of minimally processed fresh cut vegetables

1.2.1.1 Microbial safety

Increased prevalence of food borne illness in recent year world over has been associated with the consumption of fresh produce. Of this 35-40% was linked to the E. coli outbreak from consumption of leafy green vegetables such as iceberg lettuce and spinach [19]. Hence, maintenance of microbial quality forms an important aspect of post harvest storage of leafy green vegetables. Food and Agriculture Organization and United Nations World Health Organization, 2008, concluded that from a global perspective, leafy green vegetables currently presented the greatest concern in terms of microbiological hazards [20]. Such 'ready-to-use' (RTU) vegetables retain much of their indigenous microflora after minimal processing. Pathogens may form part of this microflora, posing a potential safety problem. Several studies have been carried out to determine the survival/growth characteristics of pathogens on a range of RTU vegetables. Minimally processed vegetables harbour large population of microorganisms and counts of 10^5-10^7 CFU g^{-1} are generally present [21]. Damage of fresh produce during processing operations renders them susceptible to contamination via growth/survival of spoilage or pathogenic bacteria. 80-90 % of bacteria are Gram negative rods predominantly *Pseudomonas*, *Enterobacter* or *Erwinia* species. Yeasts commonly isolated include *Cryptococcus, Rhodotorula, Candida, Aureobasidium, Fusarium, Mucor, Phoma, Rhizopus* and *Penicillium*. Pathogenic bacteria may include *Listeria monocytogenes, Claustridium botulinum, Aeromonas hydrophila, Escherichia coli, Salmonella, Yersinia enterocolitica,* and *Campylobacter jejuni.* Table 4 provides the limits of microbial population for minimally processed mixed salad vegetables.

Table 4 Officially recommended microbiological data for prepared mixed salad vegetables [22].

	Limit g^{-1}
	$< 5 \times 10^5$
TOTAL COUNTS AT PRODUCTION	
TOTAL COUNTS AT RETAIL	$< 5 \times 10^7$
RECOMMENDED SHELF LIFE	7 days including the day of production

1.2.1.2 Appearance

When assessing plant product quality, consumers consider product appearance into consideration as a primary criterion with color being the main factor in their assessment. Preservation of color is an important factor after safety as frequently a product is selected for its appearance, in particular, its color. Color has been considered to play a key role in food choice, food preference and acceptability, and may even influence taste thresholds, sweetness perception and pleasantness [23]. A wide range of factors influences appearance right from wound-related effects like browning to microbial colonization.

Browning

Browning is associated with the loss of cellular integrity and de-compartmentalization in response to cellular injury [24,25]. It mainly involves metabolism of phenolic compounds. In intact plant cells phenolic compounds in cell vacuoles are spatially apart from the oxidizing enzymes present in the cytoplasm. Once tissues are damaged by cutting, grinding or pulping, the mixing of the enzymes and phenolic compounds as well as the easy oxygen diffusion to the inner tissues result in a browning reaction. Browning also leads to off flavors and losses in nutritional quality. Unlike typically processed foods, fresh-cut products consist of living tissues that sustain considerable injury during processing. In response to tissue injury phenylalanine ammonia lyase (PAL) produces phenols which are then oxidized by polyphenol oxidase (PPO) and peroxidase (POD) to o-quinones that further polymerize to brown pigments [24,25] (Fig 2).

Enzymes involved in enzymatic browning

PAL is the first enzyme in the phenylpropanoid pathway involved in synthesis of phenylpropanoids. Phenylpropanoids serve a range of functions in plants. They act as structural components (such as lignin), protectants against biotic and abiotic stresses, antipathogenic agents (phytoalexins), antioxidants, UV-absorbing compounds, pigments (particularly the anthocyanins) and signalling molecules (e.g. flavonoid nodulation factors) [26]. Deamination by phenylalanine ammonia-lyase forms the phenylpropanoid skeleton, producing cinnamic acid. Cinnamic acid 4-hydroxylase (C4H) catalyzes the introduction of a hydroxyl group at the para position of the phenyl ring of cinnamic acid, producing p-coumaric acid. The carboxyl group of p-coumaric acid is then activated by formation of a thioester bond with CoA, a process catalyzed by p-coumaroyl:CoA ligase (4CL). Subsequent reactions produce several phenolic compounds, among which 5-caffeoylquinic acid (chlorogenic acid), 3,5-dicaffeoylquinic acid, caffeoyltartaric acid and dicaffeoyltartaric acid have been associated with increased browning in vegetables [28].

Various stresses, such as nutrient deficiencies, viral, fungi, and insect attack are known to increase either PAL synthesis or activity in different plants [24]. Numerous reports have demonstrated a positive correlation between increased enzyme activity, PAL protein accumulation and PAL gene expression. The PAL gene is also differentially expressed during development. Several studies have described a relationship between PAL activity and browning in cut tissues. A consequent increase in phenolic content due to increase in PAL activity and a resultant increase in o-quinone content leads to browning in cut tissue upon storage. It has been proposed that this increase in PAL activity could be used as a predictive index of shelf life [27,28]. An increased PAL

activity has also been correlated with a decrease in shelf-life and overall visual quality of minimally processed lettuce [27,28].

PPO, a copper-containing enzyme ubiquitous in plants, is a generic term for the group of enzymes that catalyze the oxidation of phenolic compounds to produce brown color on cut surfaces of fruits and vegetables [24,25]. These enzyme insert molecular oxygen in ortho position to an existing hydroxyl group in an aromatic ring, followed by the oxidation of the diphenol to the corresponding quinone. The structure of the active site of the enzyme, in which copper is bound to six or seven histidine residues and a single cysteine residue is highly conserved. Like PAL, PPO is also known to be sensitive to several biotic and abiotic stresses [24]. Wounding and herbivore attack have been shown to induce PPO activity. PPO is often implicated in plant defence against the stress, but the underlying mechanism is not well investigated. Signalling molecules like methyl jasmonate is often known to induce PPO gene expression in plants but this induction, is by no means a universal response [29]. Cutting of fresh vegetables and fruits often lead to an increase in PPO activity resulting in browning reactions in these products [24,25].

Peroxidases, another quinone forming enzyme, can oxidize both mono and diphenols to o-quinones in the presence of small amounts of hydrogen peroxide [24,25]. However, the role of POD in browning reaction remains questionable, being limited to availability of hydrogen peroxide. Apart from phenol oxidation, peroxidases have been implicated in several other primary and secondary metabolic functions which includes the regulation of cell elongation, cross-linking of cell wall polysaccharides, lignifications, wound-healing, pathogen defence. It is not known whether the induction

of peroxidase activity in response to wounding in plants is an effect of transcription rate, translational control, or related to heme availability.

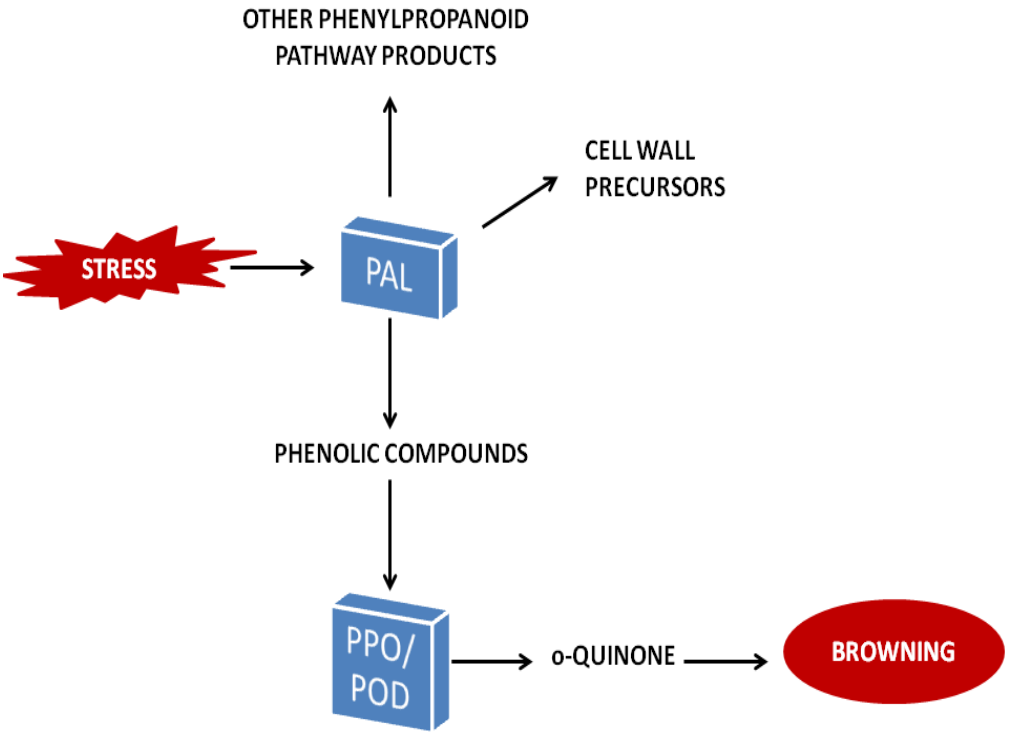


Fig 2 Wound induced enzymatic browning

1.2.1.3 Texture

Texture of a food sample can be defined as group of physical characteristics that arise from the structural elements of the food, sensed by the feeling of touch, are related to the deformation, disintegration and flow of the food under a force, and are measured objectively by functions of mass, time, and distance [30]. Consumers generally have clear preconception about the texture of fresh-cut vegetables. For example, salad vegetables like lettuce, carrot, celery, and radish are expected to be crisp. On the other hand undesirable textural attributes such as wilted lettuce, limp carrots or celery, and flaccid radish are unacceptable for the consumers.

In processed fruits and vegetables, changes in texture are strongly related to transformations in cell wall polymers due to enzymatic and non-enzymatic reactions. Cell walls are composed of cellulose, hemicelluloses, pectic substances, proteins, and also lignins in the case of vegetables. Cellulose and hemicellulose show minimal changes in structure and composition in most plant based foods [31]. Most of the changes observed in plant based foods are ascribed to transformations in pectin structure and composition. These changes are strongly influenced by the processing steps and conditions. Generally, changes in structure of pectin and thereby its composition may arise from enzymatic and/or non-enzymatic reactions. Both reaction mechanisms are imperative for processed plant based food and damaging of cells during processing operations tend to accelerate the process. Apart from mechanical injury imposed by processing operations microbial growth also bring textural changes in minimally processed vegetables during storage [32]. The rapid texture breakdown observed in cut vegetables during storage is often the result of higher aerobic psychotrophic counts. Different micro-organisms produce pectinolytic enzymes

including pectate lyase, polygalacturonase and pectin methyl esterasees resulting in textural changes. The most commonly isolated pectinolytic bacterial species are *Erwinia* and *Pseudomonas*. Pectinolytic yeasts and moulds include *Trichosporon* sp and *Mucor* sp respectively [32].

While, generally flavor is being cited as the most important quality attribute, textural defects and the interaction of flavor and texture are more likely to cause rejection of a fresh product [33]. Studies on consumer preferences together with taste panel evaluation have shown that consumers are actually more sensitive to small differences in texture than flavor.

1.2.1.4 Flavour

Among the various factors related to food palatability, flavor is the key determinant as it directly effects the perception in the mouth. Although color and appearance may be the initial quality attributes that influences consumer choice of a food product, the flavor may have the largest impact on acceptability and desire to consume it again. Flavor is a combination of aroma and taste. A diverse array of fruit and vegetable constituents including acids, sugars, volatiles and many other compounds individually elicit sensory responses that are recognized in total as flavor.

Taste

Taste can be classified into five basic categories - sweetness, sourness, saltiness, bitterness and umami. Amongst these, first four are perceived at specific areas of the tongue while umami is perceived through the whole oral cavity. Taste is the sensation produced when a substance in the mouth reacts chemically with taste receptor cells located on taste buds. Taste buds are able to differentiate among different tastes through detecting interaction with different molecules or ions. Sweet, umami, and

bitter tastes are triggered by the binding of molecules to G protein-coupled receptors on the cell membranes of taste buds. Saltiness and sourness are perceived when alkali metal or hydrogen ions enter taste buds, respectively.

Sweet taste in food is imparted by a number of compounds which are broadly classified as natural or artificial. This taste is perceived at the tip of the tongue. Sourness in food is generally imparted by acidic compounds and hence also affects the pH of the food. Food having a pH lower than 4 is considered asvery sour whereas from 4.5 to 6.5 are slightly sour. Citric acid, malic acid and oxalic acid are the acidic compounds attributing to sourness to vegetables. Sourness is perceived by the taste buds located at the sides of tongue. Saltiness is mainly due to inorganic salts like sodium chloride. Vegetables are usually known to have very low salt content with Na content 0.4 g/kg. Saltiness is perceived at the upper surface of the tongue. Bitterness is a key taste attribute generally reducing the acceptability of the food. The phenolic compounds and the isothiocyanates are known to impart bitter taste to brassica vegetables. Alkaloids and terpenes are the other bitter compounds present in food. These compounds contribute to the bitter taste in broccoli is while in some other vegetables bitterness is often masked due to the presence of other taste contributing compounds.

Apart from the other tastes, trigeminic receptors in mouth generally perceive hot (capsaicin in hot peppers, mustard oil in radish, mustards), cold (menthol, cucurbitacin in cucumber) and astringent (tannins) sensation in food. Figure 3 provides examples of compounds contributing to sensation of taste of food.

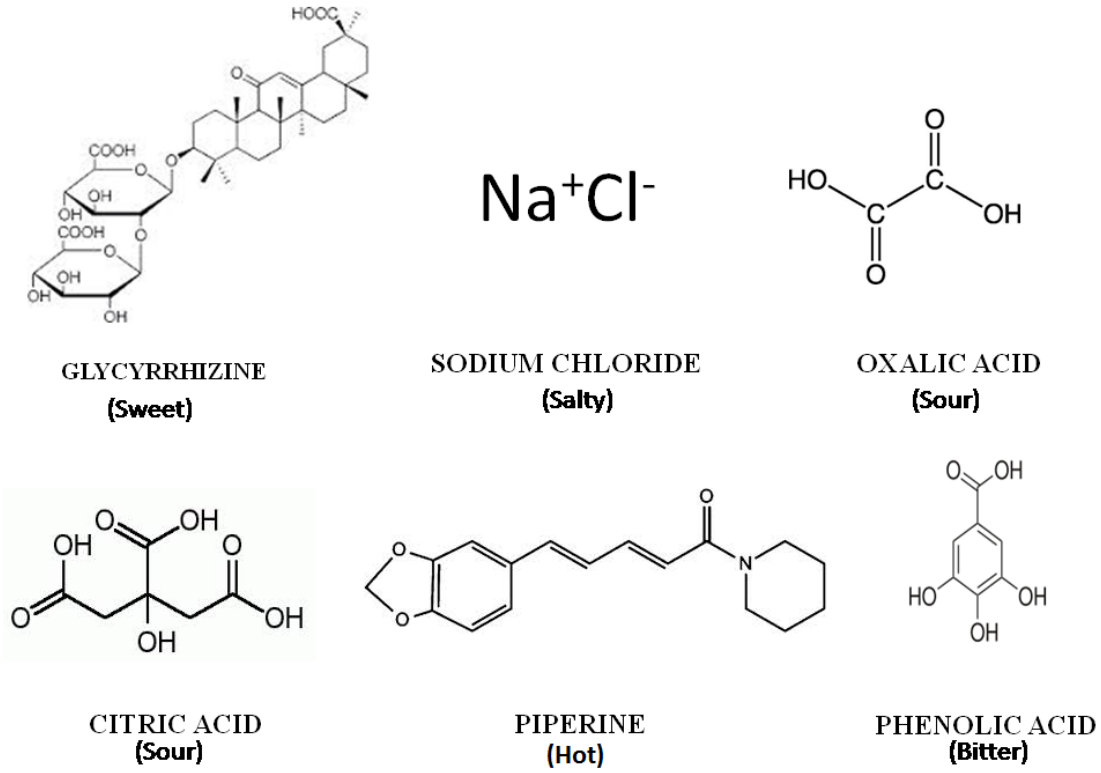


Fig 3 Taste contributing compounds in vegetables

Aroma

Vegetable aroma is the result of a unique combination of different metabolites which are volatile in nature. The different proportions of the volatile components and the presence or absence of trace components often determine aroma properties. Various factors affect the volatile profile of the vegetable including genetics, maturity, growing conditions and post harvest handling.

The amount of volatile substances present in food is extremely low (ca. 10–15 mg/kg). In general, however, they comprise a large number of components. Of all the volatile compounds, only a limited number are important for aroma. The volatile compounds associated with aroma profile of the species are known as odor active compounds and those that provide characteristic aroma of the food are called key odorants (character impact aroma compounds). The odor active compounds are present in food in

concentrations higher than the odor thresholds. Odor threshold (recognition threshold) is the lowest concentration of a compound that is just enough for the recognition of its odor. Threshold concentration data allow comparison of the intensity or potency of odorous substances. Compounds with concentrations lower than the odor threshold can also contribute to aroma when mixtures of them exceed these thresholds.

Terpenes, isothiocyanates, sulphides, thiols, C6 aldehydes and alcohols are the major volatile classes that are responsible for the characteristic odor of vegetables (Fig 4). These compounds are biosynthesized mainly from the three primary metabolites namely carbohydrates, amino acids and lipids. These pathways will be discussed in later section.

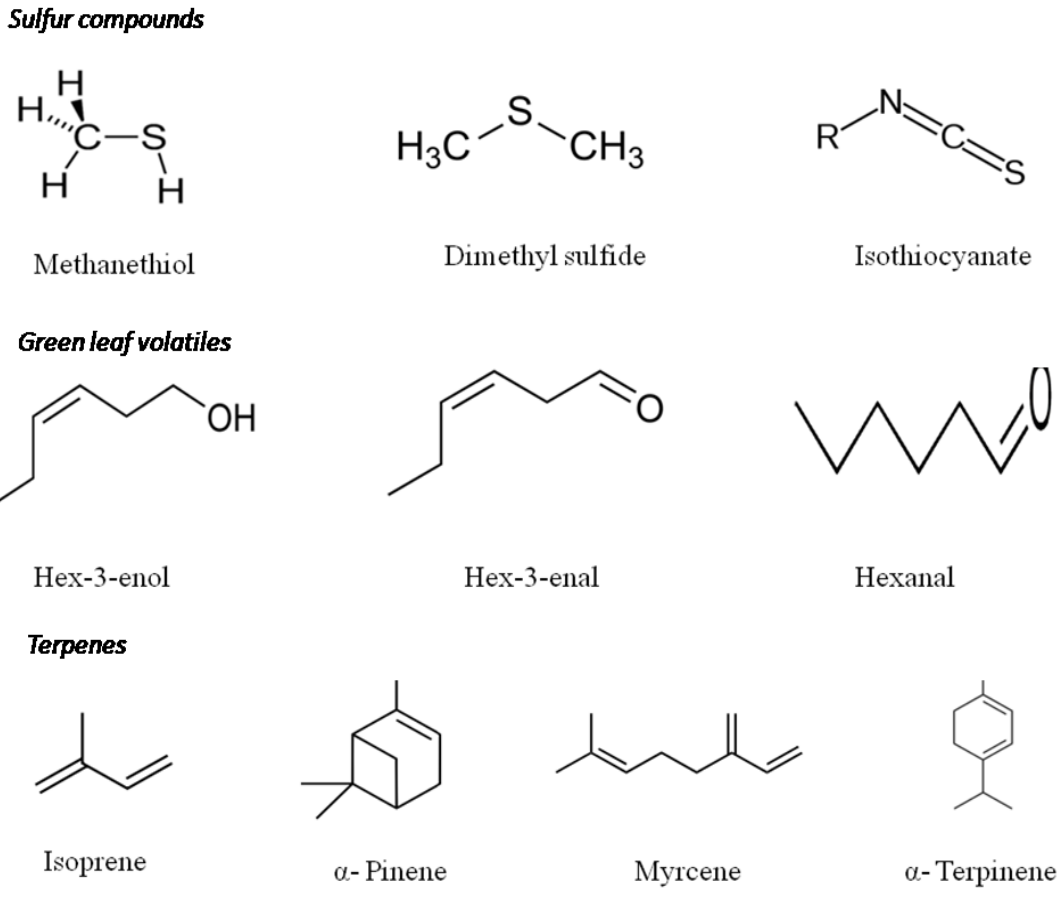


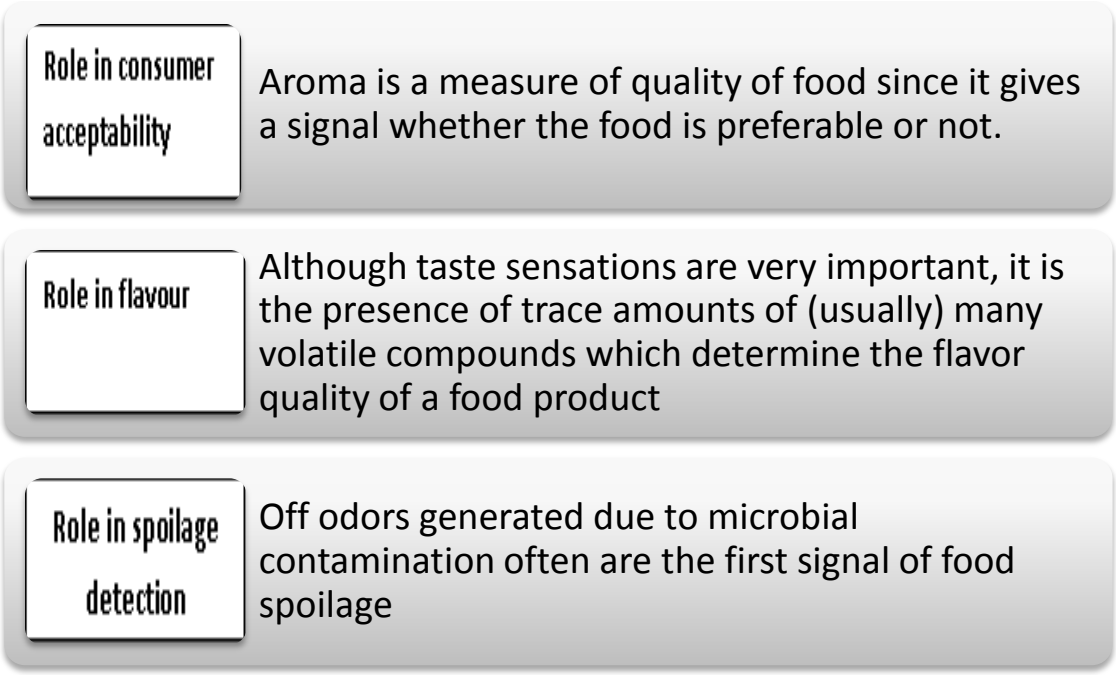
Fig 4 Aroma compounds of vegetables

Table 5 Aroma compounds present in different food stuff and the odor notes associated with it.

COMPOUND	ODOR NOTE
Hydrogen sulfide	Rotten egg
Methanethiol	Sulfurous, putrid
Ethanethiol	Onion
Dimethyl sulfide	Sulfurous
Diethyl sulfide	Ether
Diethyl disulfide	Garlic
Ethyl acetate	Fruity
Propyl acetate	Fruity
Hexanal	Herbaceous
Hex-2-enal	Green
Hex-3-enol	Green
Hexenyl acetate	Green
Octanal	Sweet, honey like
Nonanal	Fatty-floral
Decanal	Sweet, waxy
3-Methyl butanal	Fruity, malty
Limonene	Citrus like
2,3-Butanediol	Buttery
Guiacol	Smoky
Isothiocyanates	Sulfurous

Modified after Olusola Lamikanra, Fresh-Cut Fruits and Vegetables: Science, Technology

Importance of aroma in food



1.2.2 Quality evaluation of minimally processed vegetables

The quality of food products including fresh-cut produce is normally measured by both subjective and objective analysis. Subjective methods usually involve assessment of sensory quality of the product by a panel of human assessors. Objective analysis on the other hand, involves use of analytical instruments for assessing the quality of the food product. The main advantage of subjective analysis over instrumental analysis is that the quality attributes can be clearly defined in terms that are relevant to consumer acceptability as it involves human perception. The benefit of subjective evaluation results from the fact that no instrument has the ability to imitate human senses; hence, use of human assessors is the best way to evaluate the quality of a product. When carefully coordinated, the subjective tests can be very effective in developing new products and establishing quality standards. However, subjective methods require extensive training and can produce highly variable results if training is inadequate.

The results from consumer panels tend to be highly variable. On the other hand, instrumental techniques are advantageous in that they tend to provide accurate and precise results. The results of instrumental tests can generally be related directly to chemical and physical properties allowing the investigator to gain a mechanistic understanding of observed differences. Instrumental tests are more useful in measuring standards in a quality control setting. In general, subjective and instrumental tests are best used in conjunction with each other using the most appropriate test to meet the desired objective.

1.2.2.1 Instrumental evaluation

Instrumental methods of measuring appearance, color, texture, aroma, and flavor in fruits and vegetables were first described by Kramer [35], and later amended by Kader [36]. A modified list of methods of quality measurement is depicted in Table 6.

Table 6 Instrumental methods for determination of vegetables quality

Quality Attribute	***Objective method of measurement***
Color	Color charts, reflectance and transmittance colorimeters, pigment extraction and spectrophotometers
Texture	Texture analyzers-compression, shearing, analysis of solids
Aroma	Gas chromatograph, enzymes
Nutritional value- Antioxidants, Vitamin A, B, C, E, polyphenolics, carotenoids, glucosinolates	HPLC and spectrophotometric methods

Evaluation of color

Color may be determined using nondestructive methods based on visual or physical measurements. Instrument analysis of color is done by using either colorimeters or spectrophotometers. Colorimeters give measurements that can be correlated with human eye-brain perception [37]. Spectrophotometers provide wavelength-by-wavelength spectral analysis of the reflecting and/or transmitting properties of objects, and are more commonly used in research and development laboratories [37]. Commission Internationale de l'Eclairage (CIE) or International Commission on Illumination governs the measurement of color. Color space may be divided into a three-dimensional (L, a and b) rectangular area such that L (lightness) axis goes vertically from 0 (perfect black) to 100 (perfect white) in reflectance or perfect clear in transmission [37]. The "a" axis (red to green) considers the positive values as red and negative values as green; 0 is neutral. The "b" axis (blue to yellow) expresses positive values as yellow and negative values as blue; 0 is neutral. Pigments of vegetables may also be analyzed quantitatively by extraction with specific solvents, filtration, and the use of various methods based on spectrophotometry. Separation using reversed phase high performance liquid chromatography (HPLC) may be useful prior to measurement of absorption of light in the uv/visible spectrum.

Analysis of Texture

The instrumental analysis of texture of fresh cut vegetables is primarily concerned with the evaluation of mechanical characteristics of the product and is usually carried out using texture analyzer. This instrument applies a wide range of simple and rapid tests, including puncture, compression, extrusion, shear, and others, which measure one or more textural properties and are commonly used in quality control applications.

The texture analyzer measures the amount of force resisting the deformation by a sample.

Analysis of flavour

Analysis of flavour of fresh cut vegetable mainly involves analysis of aroma and taste of food sample. Sweetness can be approximated by HPLC determination of individual sugars, by a refractometer or hydrometer that measures total soluble solids [35]. Indicator papers are used for rapid determination of glucose [38]. Chloride and/or sodium content is usually estimated as an approximation of saltiness. Sourness is determined by measuring either pH or total acidity of the sample [39]. Both indicator papers and pH meters are available for the determination of pH [39]. The total acidity is measured by titration methods. Finally, astringency may be indicated by measuring total phenolics and bitterness by analysis of compounds such as alkaloids or glucosides using HPLC [39]. Pungency is normally also measured subsequently using *Scovelli* heat units.

Analysis of aroma of food is a complex procedure involving isolation, identification and quantification of aroma compounds. Aroma isolation from a given matrix involves crushing, homogenizing, blending or extracting the matrix with minimum loss in these constituents [40]. Commonly used techniques are solvent-solvent extraction, steam distillation, solid phase microextraction, high vacuum distillation etc [40]. Solvent extraction using organic solvents at room or sub ambient temperatures is one of the most common and conventional method for extraction of aroma compounds. The nature of the solvent used, polar or non-polar, depends on the type of compounds to be isolated and identified. Drawbacks of this method, however, are the co-extraction of non-volatile constituents posing problems in recovery of volatile odors. Steam distillation is a common method of isolation of aroma

compounds from vegetables. Isolation of organic compounds from food materials by routine distillation under atmospheric pressure causes degradation of these compounds. Use of steam in distillation results in lowering in their boiling points and allowing them to be distilled at lower temperatures thus reducing their degradation. If the substances to be distilled are very sensitive to heat, steam distillation may be applied under reduced pressure, thereby drastically reducing the operating temperatures. During distillation the vapors are condensed and allowed to mix with solvent vapor that efficiently extract the volatile in the vapor phase. Two-phase system of water and the organic solvent allows for separation of volatile of interest. Solid-phase microextraction (SPME) is a solvent less sample preparation technique involving the use of a fiber coated with an extracting phase, that can be a liquid (polymer) or a solid (sorbent). This phase has the ability to extract various analytes (including both volatile and non-volatile) from both liquid as well as gas phase. Non-polar volatile compounds are effectively extracted with nonpolar fiber coatings such as polydimethylsiloxane (PDMS) while polar volatiles can be extracted with PDMS/divinylbenzene or PDMS/Carboxene polar fibers. The quantity of analyte extracted by the fibre is proportional to its concentration in the sample when equilibrium is attained. Convection or agitation normally causes achievement of short time pre-equilibrium. After extraction, the SPME fiber is transferred to the injection port of separating instruments, such as a Gas Chromatograph, where desorption of the analyte takes place and analysis is carried out.

The extract thus obtained contains several different constituents in varying amounts. The individual components need to be separated from the mixture to facilitate their identification. The most commonly used method of separation is the chromatographic technique based on adsorption / partition of constituents between two phases. Among the chromatographic

techniques gas chromatography is the most efficient technique for the separation, identification and quantification of volatile organic compounds. It utilizes a capillary column which depending on the column's dimensions (length, diameter, film thickness), phase properties and the relative affinity of the individual compounds for the stationary phase promotes separation of the molecules as the sample travels the length of the column. Commonly used stationary phases are the non polar dimethyl polysiloxanes (DB-l, DB-5, CPSil 5, SE-30 and OV-1) and the more polar polyethylene glycol polymers (Carbowax^{TM} 20 *M*, DB-Wax and HP 20M). For different stationary phases, retention index data such as kovats index system have been developed to facilitate compound characterization and identification. Detection of peaks can be carried out using two types of detector. First type include the flame ionization detector (FID) and the thermal conductivity detector (TCD) that provide the retention times while the second type include the mass spectrometer (MS) and the Fourier transform infrared(FT-IR) spectrometers that aid in obtaining structural information. FID is a highly sensitive detector (0.05 – 0.5 ng per compound) and is based on detection of ions formed when organic compounds are burnt in a flame, while TCD, a less sensitive detector, operates by differential thermal conductivity of gaseous mixture. MS with a sensitivity of 0.1 - 1 ng per compound, relies on generation of positively charged molecules/and molecule fragments from compounds separated on the GC column. Several comprehensive mass spectral libraries (WILEY, N|IST MS data base, 1998) have been established and are currently used in EI-MS searches for tentative compound identification.

Nutritional evaluation

Fresh cut vegetables are good sources of minerals, vitamins, and some beneficial phytochemicals such as carotenoids, phenolics, and glucosinolates. The determination of the nutrients in vegetables is carried out using chemical methods following their extraction in

either water or organic solvents. Atomic absorption spectroscopy is typically used to carry out mineral analysis in food samples [37]. Vitamins may be determined following extraction using high performance liquid chromatography (HPLC) or using older methods that employ microbiological, turbidimetric, or titrimetric methods [37]. Vitamin C being the most stress sensitive vitamin, its estimation gives an indication of overall nutritional quality of the vegetable. Official AOAC method uses titrimetric and microfluorometric methods for vitamin C estimation. Antioxidant activities of different vegetable extracts are estimated by standard AOAC protocols [39].

The bioactive phytochemicals of vegetables are typically analyzed using HPLC [39]. Phenolic compounds are usually extracted in water or a water methanol mixture. Further analysis usually involves HPLC or LCMS analysis for separation and identification of the compounds. In the case of colored phytochemicals like anthocyanins, it is possible to estimate its content by measuring the intensity of color or a/b value with a colorimeter, but such a physical method is not available for most nutrients. Glucosinolates generally require hot aqueous alcohols such as methanol: water (70:30) for their isolation from plant materials in order to prevent their hydrolysis by myrosinase [41]. A prior separation into groups normally precedes their identification and quantification by HPLC-$MS^{n.}$ Presence of sulfate groups facilitates binding of these compounds to an anion exchange column and thus allows separation of either the intact GSLs or "desulfo" derivatives after enzymatic desulfation [41]. Direct analysis of volatile isothiocyanates and nitriles produced from GSLs by GC/MS can also provide proof of the presence of corresponding GSL in intact plant.

1.2.2.2 Subjective evaluation- Sensory analysis

Sensory analysis (or sensory evaluation) is a scientific discipline that applies principles of experimental design and statistical analysis to the use of human senses (sight, smell,

taste and touch) for the purposes of evaluating consumer products. The discipline requires panels of human assessors, who assess the products are tested and their responses are recorded. By applying statistical techniques to the results it is possible to make inferences and insights about the products under test.

Sensory evaluation of food products is divided into two components—analytical and affective measurements [39]. Analytical measurements are used to detect differences (difference tests) or to describe the product (descriptive analysis). Affective measurement gives an indication of preference, liking or acceptance of a product. Amongst the analytical and affective methods, quality descriptive analysis (QDA) and hedonic test is the most commonly applied method for analysis of fresh cut vegetables.

Qualitative descriptive analysis

QDA method of sensory analysis involves development of a panel of members based on preliminary experiments to form the trained sensory panel [42]. The sensory attributes of the food sample, usually ranging from 5 to 50, are identified by the panel. Upon identification or determination of the attribute, the panel must go through training and calibration to ensure that the panel results are accurate and precise. Upon the completion of training, the evaluation of the samples is conducted in partitioned booths. Samples are analyzed and each of the attribute is quantified by marking on an unstructured scale of 15 mm with the scale from very weak to very strong from left to right [43]. The sensory data are collected by measuring from extreme left hand point of the scale. Finally based on analysis a spider diagram is constructed depicting the judgement of the panel.

Hedonic testing

The most commonly used technique to measure the likeness of a food sample is hedonic testing. The term hedonic means "having to do with pleasure". Consumer analysis for fresh cut vegetables is done by this method. Hedonic test generally requires a large number of untrained respondents to obtain an indication of appeal of one product versus another. This test involves marking different sensory attributes in a food sample from 1 to 9; where 1 represents dislike extremely and 9 like extremely [44]. The data are then analyzed by t test in case of 2 samples and ANOVA for multiple samples.

1.2.3 Approaches in improving shelf life of minimally processed vegetables

The main objective of food industry in improving shelf life of minimally processed vegetables is preservation of sensory and nutritional quality of the product while maintaining the microbial safety of the product. A number of processing techniques are applied for post harvest shelf life enhancement which can be broadly divided into chemical and physical methods and a combination of the two *i.e.* the hurdle technology.

1.2.3.1 Chemical preservatives

Chemical preservatives are usually applied during washing of the cut products. The preservatives can be used in the wash water to reduce microbial population and retard enzymatic activity, thereby improving both the shelf life and sensory quality of the product. According to several researchers, 100-200 mg of chlorine or citric acid per litre in the wash water is effective before or after peeling and/or cutting to extend shelf life [45]. However, when chlorine is used, vegetable material should subsequently be rinsed to reduce the chlorine concentration to that found in drinking water and to

improve the sensory quality. Recent studies have shown, chlorine dioxide to be a better oxidating agent than chlorine [46]. Hydrogen peroxide, a strong oxidizing agent and ozonated water is also used for reducing microbial populations and shelf life extension of fresh produce [47]. Efficacy of ClO_2 in the inactivation of *Listeria monocytogenes* and *Salmonella Typhimurium* and H_2O_2 solution in reducing microbial populations on fresh-cut bell peppers, cucumber, zucchini, cantaloupe, and honeydew melon, without alteration in sensory characteristics have been reported [46]. Although, antimicrobial activity of ozone is widely known, there is little information available about its efficacy against food borne pathogens like *Shigella sonnei*. Higher corrosiveness of ozone and initial capital cost for its generation are the main disadvantages in its use compared to other chemical preservatives.

In the case of products, like sliced potatoes, for which the main quality problem is browning, anti-browning agents are usually added to the washing water. Citric acid combined with ascorbic acid is one such additive [24]. However, these anti-browning agents being reducing in agent often act antagonistically to most of the sanitizers used for controlling microbial load which are oxidizing in nature. Consequently, in combination they generally cancel out each other's desired effects [24].

Calcium is another additive frequently used for shelf life extension of fruits and vegetables [48]. It chelates with pectin of cell wall to form calcium pectate thus maintaining the cell wall's integrity. Different salts of calcium used for food preservation include calcium chloride, calcium lactate and calcium propionate. Amongst these calcium propionate also has the ability to uncouple microbial transport processes thus acting as a potent bactericide.

Acidic electrolyzed water (pH 2.1-4.5) has a strong bactericidal effect against pathogens and spoilage microorganisms [49]. It is more effective than chlorine due to its high oxidation-reduction potential (ORP). A higher effectiveness of electrolyzed water in reducing viable aerobes than ozone on whole lettuce has also been demonstrated. No adverse effects were noted on surface color, pH or general appearance of fresh-cut vegetables.

Reluctance of consumers towards the use of chemical preservatives in recent years has resulted in the use of natural antimicrobials as preservatives. Organic acids such as lactic, citric, acetic and tartaric acids are used as strong antimicrobial agents against psychrophilic and mesophilic microorganisms in fresh-cut fruits and vegetables. The antimicrobial action of organic acids is due to pH reduction in the environment, disruption of membrane transport and/or permeability, anion accumulation, or a reduction in internal cellular pH by the dissociation of hydrogen ions from the acid.

1.2.3.2 Physical methods of preservation

Minimally processed fruits and vegetables are preserved by several physical methods that include modified atmosphere packaging, refrigeration, mild heat treatments, microwave processing, ionizing radiation, high pressure technology, high intensity pulsed electric field, pulsed light etc [50]. Amongst these modified atmosphere packaging forms one of the most studied method. The basic principle in MAP is to create a modified atmosphere either passively by using appropriately permeable packaging materials, or actively by using a specified gas mixture together with permeable packaging materials. Both the principles aim to create an optimal gas balance inside the package, where the respiration of the product is low, but the levels of

oxygen and carbon dioxide are not detrimental to the product. In general, the aim is to have a gas composition of 2-5% CO_2, 2-5% O_2, and the rest nitrogen.

Ultraviolet (UV) light is another physical treatment widely employed in industry[50]. UV irradiation causes up to 4 log cycle reduction in bacterial, yeast and viral counts by inducing DNA damage. Major advantage of this technique is the availability of relatively inexpensive and easy to use equipment. Among the other technologies, treating products with millisecond pulses (1–20 flashes/sec) of broad spectrum white light, about 20,000 times more intense than sunlight holds promise. Pulsed white light inactivates microorganisms by combination of photochemical and photothermal effects, requires very short treatment times and has a high throughput. The above methods, however, have lower efficiencies due to their lower penetration and are thus mostly used for surface sterilization.

1.2.3.3 Radiation processing as a promising technology for post harvest shelf life improvement

Food irradiation is a physical means of food processing involving exposure of food products to gamma rays, X-rays, or electron beam for eliminating disease-causing microorganisms [51]. It is one of the most extensive and thoroughly studied methods of food preservation. Being a cold process it can efficiently decontaminate or sterilize food without significantly affecting its sensory and nutritional quality. The non-residual feature of ionizing radiation is a significant advantage minimizing the use of chemicals applied to fruits and vegetables. In 1980, Joint Expert Committee of Food and Agriculture Organization / International Atomic Energy Agency / World Health Organization on Food Irradiation FAO/IAEA/WHO, 1981 concluded “The irradiation treatment of any food commodity up to an overall average dose of 10 kGy present no

radiological, microbiological or toxicological hazard" [52]. As a result toxicological testing of foods so treated is no longer required. Food irradiation is now legally accepted in many countries.

The irradiation technology is approved by FAO/IEAE/WHO joint committee on wholesomeness of food and currently this technology is commercially practiced in several countries [53]. The Codex Committee on Food Standards of the Codex Alimentarius Commission has also revised in 2003 the Codex General Standard for Irradiated Foods that sets standards for process foods world-wide. In 1994 Government of India amended Prevention of Food Adulteration Act (1954) Rules and approved irradiation of onion, potato and spices for domestic market. Additional items were approved in April, 1998 and in May 31 2001. In 2004 the government amended plant protection and quarantine measures. Laws and regulations enacted under the Atomic Energy Act enforced by the Atomic Energy Regulatory Board, an independent body, governing operations of irradiators used to process products, such as medical supplies as well as food. Many medical product irradiators are operating in India and around the world. Only those foods approved under the Prevention of Food Adulteration (PFA) Act rules can be irradiated and sold in domestic market (Table 7). Food irradiation has been considered a safe and effective technology by the World Health Organization (WHO), the Food & Agriculture Organization (FAO), and the International Atomic Energy Agency (IAEA) [54]. The process has recently been recommended for microbial decontamination of fresh leafy green vegetables of the Brassica species such as spinach and lettuce [55]. FDA, USA allows the use of ionizing radiation up to 4 kGy to make these products safer and delay spoilage [56]. This has lead to a greater interest in the use of radiation processing particularly for vegetables of Brassica family.

Table 7 Food items approved for irradiation in India under PFA act rules

Name of the food	Purpose	Dose (kGy)	
		Min	Max
Onion	Sprout inhibition	0.03	0.09
Potato		0.06	0.15
Ginger, garlic		0.03	0.15
Shallots		0.03	0.15
Mango	Disinfestation (Quarantine)	0.25	0.75
Rice, Semolina, Whole wheat flour	Insect disinfestation	0.25	1.00
Raisins, figs and dried dates		0.25	0.75
Pulses		0.25	1.00
Dried sea-foods		0.25	1.00
Meat and meat products including chicken	Shelf-life extension and pathogen control	2.50	4.00
Fresh sea-foods	Shelf-life Extension under refrigeration	1.00	3.00
Frozen sea-foods	Pathogen control	4.00	6.00
Spices	Microbial decontamination	6.00	14.00

The Prevention of Food Adulteration Act & RuleS

The potential application of ionizing radiation in food processing is based mainly on the fact that ionizing radiations by its direct effect on macromolecules and indirect effect through radiolysis of water damage very effectively the DNA thereby inactivating living cells including microorganisms, insect gametes etc [51]. The gamma irradiation may be employed for inhibition of sprouting, delay in ripening, killing of insect pests, parasites, pathogenic and spoilage microorganisms.

Application of gamma irradiation:

LOW DOSE APPLICATION (10 Gy -1 kGy)	20 -150 Gy: Inhibition of sprouting of bulbs, tubers, rhizomes and root crops by doses in the. 0.1- 1 kGy: Delay in ripening and senescence of fruits and vegetables 0.2- 1 kGy: Insect disinfestation
MEDIUM DOSE APPLICATION (1-10 kGy)	Reduction in spoilage causing micro-organisms and elimination of pathogens of different food products like fresh meat and seafood, as well as vegetables and fruits enhancing the shelf life of the product.
HIGH DOSE APPLICATION (10-100 kGy)	Total eradication of microrganisms in products like spices (10-30 kGy). Radiation sterilization(25 - 70 kGy) extends the shelf life of precooked or enzyme inactivated food products in hermetically sealed containers almostindefinitely at ambient temperature.

Advantages of gamma irradiation over other processing techniques

- Elimination of pathogens like *Salmonella, Listeria, Campylobacter, Shigella, Yersinia, Shigella, E coli O 157*
- Being a cold process, it does not alter the fresh-like character of a food commodity and at recommended doses maintains sensory qualities, texture, nutritive value and appearance of food.
- As technology can be applied to packaged food in the final retail stage form, chances of re-contamination during transportation and distribution is prevented.
- Due to its non-residual nature, it does not produce any toxic residues in food.
- Being highly penetrating and effective, large volumes of foodstuffs can be treated very efficiently.
- Radiation processing is an eco-friendly treatment and does not pollute environment.

1.2.3.4 A combinational approach: Hurdle technology

Hurdle technology is the combined use of several preservative methods at lower intensity to make the product shelf stable, to improve quality and to provide additional safety. Complex interactions of various factors such as temperature, pH, water activity, MAP and antimicrobials are employed to design series of hurdles to ensure microbial safety of food products.

Use of hurdle technology with irradiation as one of the hurdles for control of microorganisms and extending shelf life of minimally processed produce has shown considerable potential for commercial exploitation. Efficacy of gamma irradiation in combination with other preservation techniques like MAP for reducing the microbial

population and extension of shelf life while maintaining nutritional quality of minimally processed vegetables has been demonstrated.

1.3 Brassica vegetables and their importance

Among the various groups of vegetables, brassica species are one of the most popular vegetables consumed throughout the world. These cruciferous vegetables have unique tastes and aromas but also come with both significant nutritional and health benefits [57]. Additionally, *Brassica* species and varieties are increasingly becoming a research model in plant science, as a consequence of the importance of their primary and secondary metabolites.

Brassica is a genus of plants in the mustard family (Brassicaceae) collectively known as cruciferous vegetables. Common types of Brassica vegetables consumed as food include cabbage, cauliflower, broccoli, and Brussels sprouts. Almost all parts including the root (rutabaga, turnips), stems (kohlrabi), leaves (cabbage, collard greens), flowers (cauliflower, broccoli), buds (Brussels sprouts, cabbage), and seeds (many, including mustard seed, and oil-producing rapeseed) of some species or other are used as food.

Unlike other vegetables, *Brassica* vegetables are known to have high fat and protein contents and thus contribute to oil and protein requirement for human nutrition. As a part of normal diet a standard portion (100g) of crucifer vegetable can contribute, on an average, around 5-6 % of the Recommended Dietary Allowance (RDA) for energy [58]. Cruciferous vegetables also contain an appreciable level of dietary fiber that represents as much as 25-35% of the dry matter in the crops [59]. Appreciable levels of the polyunsaturated fatty acids (PUFAs) including linoleic and gamma-linolenic acids have also been reported in Brassica vegetables [60]. These compounds have

beneficial effects in reducing the risk of incidence and progression of chronic diseases like type II diabetes and coronary heart disease (CHD) [61].

Cruciferous vegetables are also a good source of vitamins including carotenes, tocopherols, vitamin C, and folic acid (Table 8). Lutein has also been isolated from extracts of fresh raw kale (*Brassica oleracea* var. *acephala*) and high levels of other carotenoids, mainly β-carotene, were also detected. Two other vegetables, Brussels sprouts and green cabbage, have been reported to contain significant amounts of *trans*-β-carotene and *cis*-β-carotene. The predominant tocopherol in all *Brassica* vegetables is α-tocopherol, the exception being cauliflower that predominantly contains γ-tocopherol. High levels of vitamin C have been reported in Chinese cabbage, broccoli, cauliflower and cabbage. Its content in different cultivars of cabbage (*Brassica oleraceae* L.) has been found to range from 12.0 to 112.5 mg/100 g [62]. Raw broccoli, cauliflower and cabbage contain appreciable amounts of folic acid. The acid reduces the risk of neural tube defects and is associated with the reduced risk of vascular disease and cancer [63]. Low folate intake has been recognized as a main cause of anaemia.

Brassica plants are rich in many minerals including calcium and iron. Among the green leafy vegetables, *B. oleracea* L. *acephala* (kale) is an excellent source of minerals, with high levels of P, S, Cl, Ca, Fe, Se, and K [64]. Broccoli can accumulate Se at concentrations several times above that found in soil, thus enhancing its health-promoting properties [65]. High mineral contents have been reported in different *Brassica* vegetables such as cauliflower, bok choy (*B. rapa*) stems and leaves, broccoli (*B. oleracea v. botrytis*), and kale (*B. oleracea v. acephala*) [66]. These vegetables also exhibit excellent calcium bioavailability. Potentially useful amounts of

copper, zinc, iron, and a number of other essential minerals and trace elements are also reported in cabbage leaf [59].

Fructose, glucose, and sucrose are the major soluble sugars found in Brassica with fructose accounting for 45-65%[66]. Dietary fibers that are non-starch polysaccharides are an important constituent of Brassicaceae vegetables contributing to the prevention of colon cancer. They account for one-third of the total carbohydrate content in white cabbage (*B. oleracea* var. *capitata*) [67]. The average total dietary fiber content of 6 cultivars of white cabbage (*B. oleracea* var *capitata*) was estimated to be 241 mg/g of dry matter while for yellow-seeded *B. napus* and brown-seeded *B. napus*, the fiber content was found to vary between 271 and 352 mg/g respectively [68]. Intermediate values were noted in in other species, such as cauliflower (302 mg/g of D.W.), broccoli (330 mg/g of D.W.), and cabbage (226 mg/g of D.W.) [59]. Thus, Brassica vegetables form an important part of human and animal nutrition contributing immensely to the daily recommended dietary uptake.

Table 8 Proximate composition of selected Brassica vegetables

	Brocolli	**Brussel sprouts**	**Cauliflower**	**Kale**	**White cabbage**	**Turnip**
Energy (cal)	147(35)	102(24)	101(24)	167(40)	116(28)	115(27)
Carbohydrate, available	*2*	*2*	2.2	4.1	4.1	4.2
Fat, total (g)	*0.3*	*0.5*	*0.3*	0.6	0.2	0.3
Protein, total (g)	*4.6*	*1.4*	1.8	3.4	1.2	1
Starch, total (g)	*0*	*0*	0.1	0	0	0.3
Fibre, total(g)	*2.5*	*2.5*	2.3	*2*	2.1	*1.9*
Fatty acids, total (g)	*0.3*	*0.2*	0.2	0.2	0.1	0.1
Cholesterol (mg)	*0.3*	*0*	0	0	0	0
Na (mg)	*6.9*	*5*	25	5	5	4.1
K (mg)	*400*	*320*	370	320	320	300
Ca (mg)	*48*	*42*	24	42	42	35
P (mg)	*90*	*41*	50	41	40	50
Fe, total(mg)	*1.1*	*0.4*	0.6	0.4	0.4	0.3
Zn (mg)	*0.1*	*0.2*	0.5	0.2	0.2	0.2
A(µg)	*85.9*	*35.8*	0.9	765.8	5.5	6
D (mg)	*0*	*0*	0	0	0	0
E(mg)	*0.7*	*0.4*	<0.1	0.9	<0.1	0
K µg)	*110*	*220*	20	618	60	2
C (mg)	*120*	*90*	61.5	110	37.4	39.7
B2 (mg)	*0.2*	*0.16*	0.06	0.35	0.05	0.06
B1 (mg)	*0.1*	*0.11*	0.1	0.12	0.07	0.06

Modified after Bhandari et al. (2005)

1.3.2 Bioactive constituents of brassica vegetables

Cruciferous vegetables, in particular those belonging to the Brassica genus, are a good source of a variety of health-promoting and potentially protective phytochemicals. Among the phytochemicals, glucosinolates that are unique to crucuiferae as well as antioxidant phenolic compounds play a prominent role in imparting bioactive properties and thus nutraceutical value to *Brassica* vegetables. Besides, the beneficial effects have also been partly attributed to phytochemicals such as phenolics, vitamin C and carotenoids that are known to have high antioxidant potential.

1.3.2.1 *Glucosinolates*

Sulfur containing glucosides, glucosinolates (GSLs), are one of the most important phytochemical of Brassica vegetables responsible for their characteristic flavor and odor. Structurally they are anions made up of thiohydroximates containing an S-linked β-glucopyranosyl residue and an O-linked sulfate residue with a variable amino acid derived side chain.

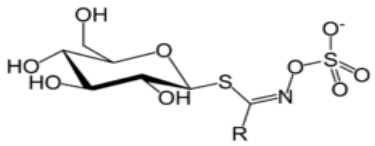


Fig 5 Glucosinolate structure

Glucosinolates represent a large chemical family consisting of over 130 different compounds with varying structural subgroups [41]. These compounds are biosynthesized from the amino acids and are involved in plant defense against pests and diseases. They have been demonstrated to possess anti-carcinogenic properties [41]. Chemoprotective action of GLS has been reported to result from modulating detoxification enzymes which protects from DNA damage and proliferation of cancer

cells [69]. These hydrophilic, stable compounds are normally sequestered in vacuoles of most plant tissues. Loss of cellular integrity initiates glucosinolate breakdown by myrosinase-catalyzed hydrolysis of the glucosidic bond. Myrosinases are specific β-thioglucosidases localized in idioblasts (myrosin cells) scattered throughout most tissues of glucosinolate producing plants.

Glucosinolates are classified into aliphatic, aromatic, and indolic forms [41]. Aliphatic glucosinolates are mostly present in *Brassica* genus while indole glucosinolates are in traces in Brassica species. The predominant glucosinolates in Brussels sprouts, cabbage, cauliflower, and kale are sinigrin and glucobrassicin while 4-methylsulfinylbutyl glucosinolate (glucoraphanin), 3-butenyl glucosinolate (gluconapin), and 3-indolylmethyl glucosinolate (glucobrassicin) are the predominant glucosinolates in broccoli [70]. Brussels sprouts also contain significant amounts of gluconapin [70]. Cruciferous vegetables of the *Brassica* genus (example, Brussels sprouts, cauliflower, and broccoli) contain high levels of an indolylmethyl glucosinolate commonly known as glucobrassicin [71]. Several glucosinolates have been identified in *B. oleracea* var. *capitata f. alba*, namely glucoiberin, progoitrin, epiprogoitrin, sinigrin, glucrafanin, gluconapoleiferin, glucoalysin, gluconapin, 4-hydroxybrassicin, glucobrassicanapin, glucobrassicin, gluconasturein, methoxyglucobrassicin, and neoglucobrassicin [72]. Table 9 lists the nature of the glucosinolates identified in some selected *Brassica* vegetables. There is a considerable interest in recent years in optimizing GSL content and composition for plant protection and human health. Glucosinolates are also responsible for the bitter acidic flavors of *Brassicacea* species. Their hydrolytic by-products such as isothiocyanates, nitriles, and thiocyanates, are responsible for the hot and pungent taste.

Table 9 Glucosinolates in different food source.

Trivial Name	R Side Chain	Food Source
Glucocapparin	Methyl	Capers
Glucolepidin	Ethyl	Radish
----	Propyl	Cabbage
Glucoputranjivin	Isopropyl	Radish
Sinigrin	2-Propenyl	Cabbage
Glucoiberin	3-Methylsulfinylpropyl	Cabbage
Glucoibervirin	3-Methylthiopropyl	Cabbage
Glucocheirolin	3-Methylsulfonylpropyl	Cow\s milk
Glucocapparisflexuosain	Butyl	Cabbage
Gluconapin	3-Butenyl	Cabbage
Progoitrin	(2R)-2-Hydroxy-3-butenyl	Cabbage
Epiprogoitrin	(2S)-2-Hydroxy-3-butenyl	Sea kale
Glucoerucin	4-Methylthiobutyl	Cabbage
Glucoberteroin	5-Methylthiopentyl	Cabbage
Glucoalyssin	5-Methylsulfinylpentyl	Rocket
4-Methoxyglucobrassicin	4-Methoxy-3-indolylmethyl	Cabbage
Neoglucobrassicin	N-Methoxy-3-indolylmethyl	Cabbage
Glucotropaeolin	Benzyl	Cabbage
Glucobarbarin	(2S)-2-Hydroxy-2-phenylethyl	Land cress
Glucosibarin	(2R)-2-Hydroxy-2-phenylethyl	White mustard

Modified after Variyar et al.(2014)

1.3.2.2 *Phenolic compounds*

Phenolic compounds is a generic term that refers to the group of compounds widely dispersed throughout the plant kingdom and characterized by having at least one aromatic ring with one or more hydroxyl groups attached (Fig 6). Phenolics are produced in plants as secondary metabolites via the shikimic acid pathway [24]. Phenylalanine ammonialyase (PAL). is the key enzyme catalyzing the biosynthesis of phenolics from the aromatic amino acid phenylalanine [24]. They can be classified based on the number and arrangement of the carbon atoms in flavonoids (flavonols, flavones, flavan-3-ols, anthocyanidins, flavanones, isoflavones and others) and non-flavonoids (phenolic acids, hydroxycinnamates, stilbenes and others) and they are commonly found conjugated to sugars and organic acids. The most widespread and diverse group of polyphenols in *Brassica* species are the flavonoids (mainly flavonols but also anthocyanins) and the hydroxycinnamic acids.

Flavonoids

Flavonoids are polyphenolic compounds comprising fifteen carbons with two aromatic rings connected by a three-carbon bridge (C6-C3-C6). They are the most abundant phenolic compounds found throughout the plant kingdom. Flavonols are the most widespread of the flavonoids. Kampferol and quercetin and to a lesser extent isorhamnetin and myricetin and their *O*-glycosides are the main flavonols reported from *Brassica* vegetables. In *Brassica* vegetables flavonoids are mainly conjugated to glucose [73]. They are also found to be acylated commonly by various hydroxycinnamic acids [73].

Within the flavonoids, anthocyanins are the most important group of colored plant pigments, possessing antioxidant activity and other useful biological properties. They

are involved in protecting the plants against excessive light and also have an important role in attracting pollinating insects. The stability, color intensity and potential biological activity of anthocyanins is determined by their chemical structure. Cyanidin is the most commonly occurring anthocyanidin reported in *Brassica* crops [73].

Phenolic acids

Phenolic acids are a kind of non-flavonoid phenolics derived from benzoic acid and cinnamic acids. They are present both in the free and esterified forms. Among these, hydroxycinnamic acids, characterized by the C6-C3 structure are the most abundant in *Brassica*. These compounds are used by plant in chemical defence. The most common cinnamic acids in *Brassica* vegetables are *p*-coumaric, sinapic and ferulic acids [74]. They are often found in conjugation with sugar or other hydroxycinnamic acids. Significant levels of hydroxycinnamic acids have been reported in *B. oleracea* species, like kale, cabbage, broccoli, and cauliflower [73]. Hydroxycinnamoyl gentiobiosides and hydroxycinnamoylquinic acids were found to be the most abundant in these crops. Recently, gallic, protocatechuic, *p*-hydroxybenzoic, vanillic, syringic, salicylic, *p*-coumaric, caffeic, ferulic and sinapic acids were identified as the most abundant in kales. Significant levels of chlorogenic acids have also been previously reported in leafy *Brassica* species, like kale, cabbage and Brussels sprouts. In a recent study on the phenolic profiles of fifteen *B. rapa* crops, including *B. rapa* var. *pekinensis*, *B. rapa* var. *chinensis*, *B. rapa* var. *oleifera*, *B. rapa* var. *ruvo* and *B. rapa* L. var. *rapa* the major phenolic compounds identified were kaempferol 3-*O*-sophoroside-7-*O*-glucoside derivatives, isorhamnetin 3-*O*-glucoside-7-*O*-glucoside, hydroxycinnamoyl gentiobioses, hydroxycinnamoylmalicacids and

hydroxycinnamoylquinic acids [74]. The most significant phenolic acids in rapeseed (*Brassica napus* var. *oleifera*) were reported to be sinapic acid derivatives, with minor amounts of *p*-hydroxybenzoic, vanillic, gentisic, protocatechuic, syringic, *p*-coumaric, ferulic, caffeic and chlorogenic acids. The main hydroxycinnamic acids of *Brassica juncea*, *Brassica carinata* and *Brassica nigra*. were malate derivatives of sinapic, ferulic, hydroxyferulic and caffeic acids [74]. Clinical studies have demonstrated that CQA can be used to decrease the absorption of glucose and reduce the body mass of overweight and obese people.

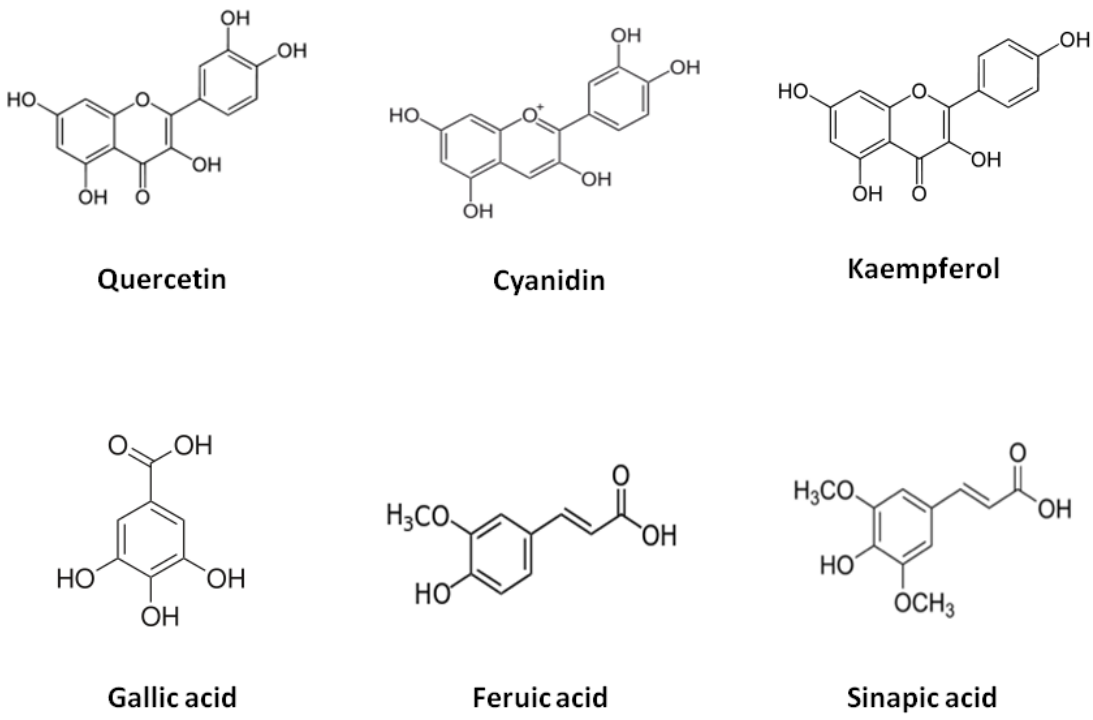
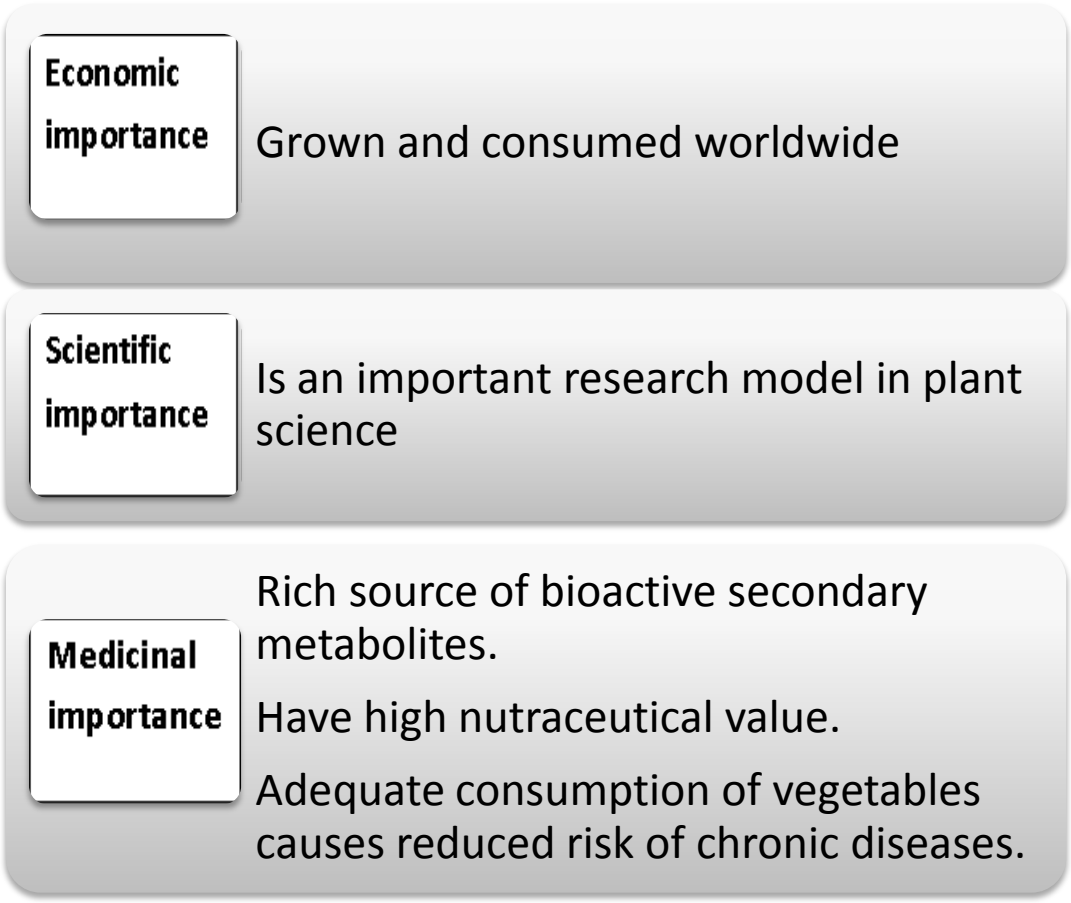


Fig 6 Phenolic compounds in brassica

1.3.2.3 *Carotenes*

Green leafy species of Brassicaceae are rich in carotenoids namely lutein and β-carotene. While the former has recently been recognized to be beneficial for eye health, the latter is well known for its pro-vitamin A activity. Among the several carotenoids identified in *B. chinensis*, *B. parachinensis*, and *B. pekinensis*, lutein and β-carotene were the most abundant [75]. Lutein as well as high levels of other carotenoids, mainly β-carotene has been isolated from extracts of fresh raw kale (*Brassica oleracea* var. *acephala*) [75]. Two other vegetables, Brussels sprouts and green cabbage, have been reported to contain significant amounts of *trans*-β-carotene and *cis*-β-carotene. Beta carotene apart from vitamin C, is one of the most important antioxidant in cruciferous vegetables. Its mean content, however, varies depending on variety, maturation phase, climate, season, agriculture practices and postharvest storage conditions. The compound is also sensitive to high temperature, particularly in acidic medium and also to oxygen and light.

Importance of Brassica vegetables



1.3.3 Aroma compounds of Brassica vegetables

Vegetables of Brassica family are characterized by their typical odor, attributed to the volatile sulfur compounds. The isothiocyanates released by hydrolysis of glucosinolates are the key sulfur compounds which produce a pungent and sulfurous aroma, playing a significant organoleptic role in brassica products [76]. Other sulfur compounds commonly reported in these vegetables are methanethiol, dimethyl sulfide, and dimethyl trisulfide [76]. Other than sulfurous aroma brassica vegetables also contain significant amount of GLVs which impart the fresh green aroma to these vegetables [77].

The important aroma imparting compounds responsible for characteristic brassica vegetables are discussed below:

Isothiocyanates : Isothiocyanates, the hydrolytic product of glucosinolates are the chief sulfurous odor imparting compounds in brassica vegetables [76]. Isothiocyanates are also known to contribute to anticancerous property of these brassica vegetables. Allyl isothiocyanate (AITC), the hydrolysis product of glucosinolate sinigrin is known to be the key odorant in many brassica vegetables like kale and cabbage [78]. This compound is reported to impart characteristic odour to these vegetables.

Dimethyl Sulfide (DMS): Reported to account for a very high proportion (~34%) of total volatiles generated in cooked Brassica vegetables. Derived from S-methylmethionine. DMS is commonly reported to impart "cauliflower aroma" to brassica vegetables [78].

Green leafy volatiles (GLVs): They comprise the volatile aliphatic C_6 compounds involving C_6 aldehydes and alcohols and their corresponding hexyl esters [77]. These compounds with their own delicately different odors, decisively contribute to these

characteristic green odors. Among the aromas of fruits and vegetables green odors are associated with the sensory perception of "natural" and produce a feeling of refreshment and relaxation.

Methanethiol (MT): Described as "putrid, fecal-like aroma" or as "cooked cabbage" is responsible for the "sulfur, cooked cabbage" of brassica vegetables [78]. It is considered to be one of the major sulfur odorants in numerous vegetables because of its low flavor threshold. Methanethiol is derived from the breackdown of S-methyl-L-cysteine sulfoxide (SMCSO) as described earlier. However, the contribution of MT to the odor of cooked Brassica has often been considered to be questionable because of its high volatility.

Terepenes: Terpenes are reported as the major volatile compounds associated with stress response in plants. A small fraction of these compounds are associated with brassica aroma [78]. The terpene limonene is reported to be a marker of the loss of green color, imparted by the green pigment chlorophyll. Other terpenes like copaene and caryophyllene are also associated with green leafy aroma of brassica vegetables.

1.3.3.1 Biogenesis of brassica aroma compounds

Aroma volatiles that impart characteristic odor to vegetables are biosynthesized from the primary metabolites such as amino acids, membrane lipids and carbohydrates [79] (Fig 7). However biogenesis of aroma compounds in vegetables differs from fruits because of the absence of ripening stage in vegetables like fruits. Most of the aroma compounds in vegetables are formed during tissue disruption. Cellular disruption causes mixing of enzymes with substrates which are spatially separated in different sub-cellular compartments in

intact cell. An important step in the biosynthetic pathway of aroma compounds is the availability of primary metabolite whose content vary widely depending on the variety and other agronomic factors.

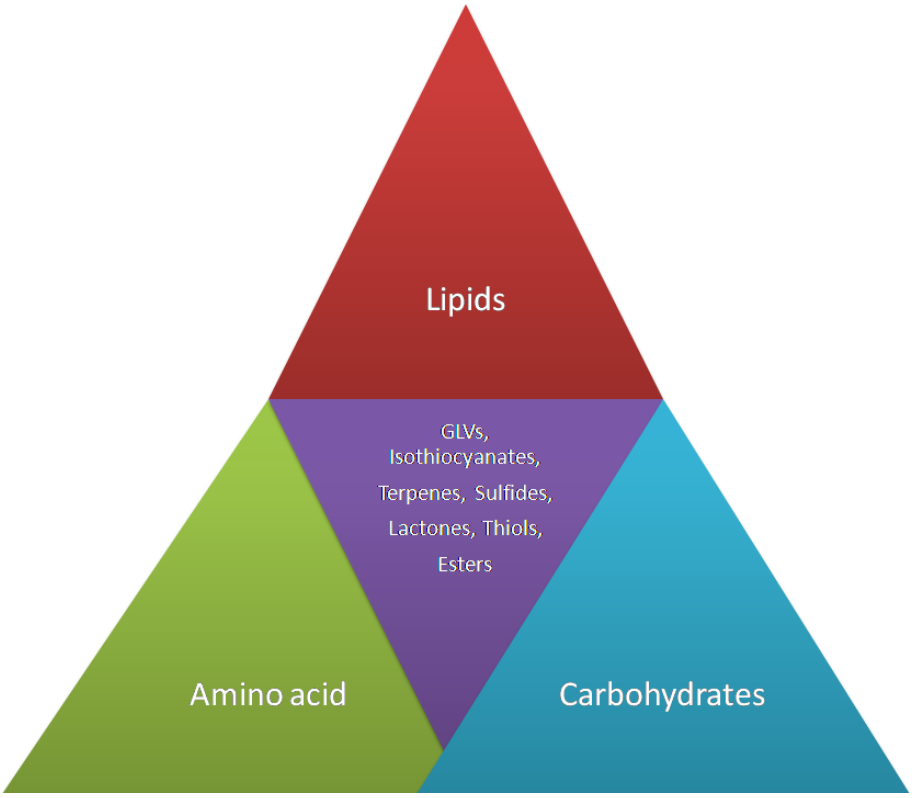


Fig 7 Biogenesis of plant aroma compounds

1.3.3.1.1 Aroma compounds derived from amino acid metabolism

A number of volatile compounds in vegetables are derived from the action of different enzymes on amino acids especially in vegetables of Brassicaceae family.

Vegetables of Brassicaceae family are characterized by typical sulfurous aroma imparted mainly by a group of sulfur and nitrogen containing compounds called

isothiocyanates. These compounds are hydrolytic products of bioactive phytochemical, glucosinolates and are responsible for the anticancerous properties of these vegetables. Glucosinolates are derived from amino acids.

Biosynthesis of GSLs involves three independent stages, namely: (1) chain elongation of selected precursor amino acids (mainly methionine) by addition of methylene groups; (2) formation of core glucosinolate structure by reconfiguration of the amino acid moiety; and (3) secondary modification of the amino acid side chain by hydroxylations, methylations, oxidations, or desaturations[41]. While the construction of core anionic structure from amino acids involves a number of common steps, a number of diverse steps are involved in formation of side chain and other modifications [85].

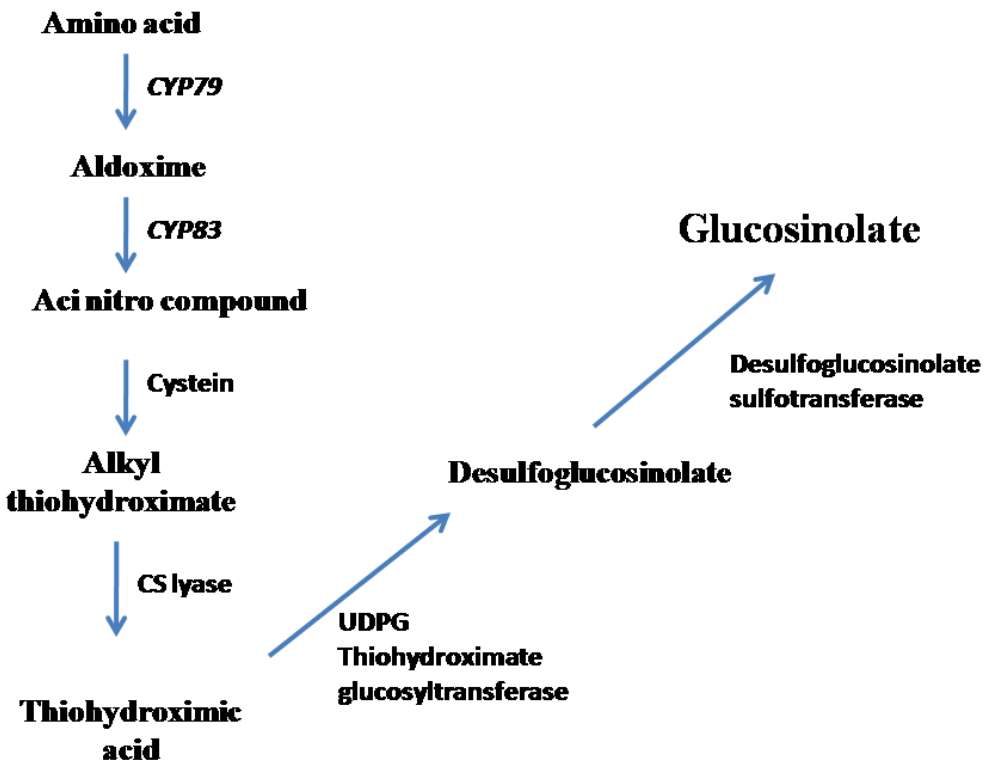


Fig 8 Biosynthesis of glucosinolate core structure

1.3.3.1.2 Aroma compounds derived from carbohydrate metabolism

Carbohydrates in brassica species are responsible for generation of mainly terpenes [79]. Terpenes constitute one of the most diverse families of natural products, with over 40,000 different structures of terpenes discovered so far. They are made up of homologous series of repetitive five carbon isoprene units in their structure. These include the monoterpenes (C10, 2 isoprene units), sesquiterpenes (C15, 3 isoprene units), diterpenes (C20, 4 isoprene units), triterpenes (C30, 6 isoprene units), tetraterpenes (C40, 8 isoprene units) and polyterpenes ([C5]n, where n may be 9-30,000). Among these, the monoterpenes and sesquiterpenes are the major constituents of several essential oils derived from odoriferous plants and plant products. Terpenes can be further sub divided into terpene hydrocarbons and oxygenated terpenes depending on the nature of their functional groups. Some of the monoterpene hydrocarbons such as myrcene, ocimene, sabinene are widely distributed in fruits and vegetables and have pleasant and characteristic odor. Oxygenated terpenes commonly exist as alcohols, aldehydes, ketones and esters.

Terpenes are enzymatically synthesized de novo from acetyl CoA and pyruvate provided by the carbohydrate pools in plastids and the cytoplasm through mevalonate pathway. Although fatty acid oxidation is one of the major pathways producing acetyl CoA, this process probably does not contribute to the formation of terpenes as its synthesis takes place in peroxisomes.

Many of the terpenes produced are non-volatile and are involved in important plant processes such as membrane structure (sterols), photosynthesis (chlorophyll side chains, carotenoids), redox chemistry (quinones) and growth regulation (gibberellins and

abscisic acid). The volatile terpenoids – hemiterpenoids (C5), monoterpenoids (C10), sesquiterpenoids (C15) and some diterpenoids (C20) – are involved in interactions between plants and insect herbivores or pollinators and are also implicated in general defense or stress responses. Monoterpenes and sesquiterpenes particularly add to flavour of fruits and vegetables. These compounds are formed through anabolic processes and are hence present in intact tissues.

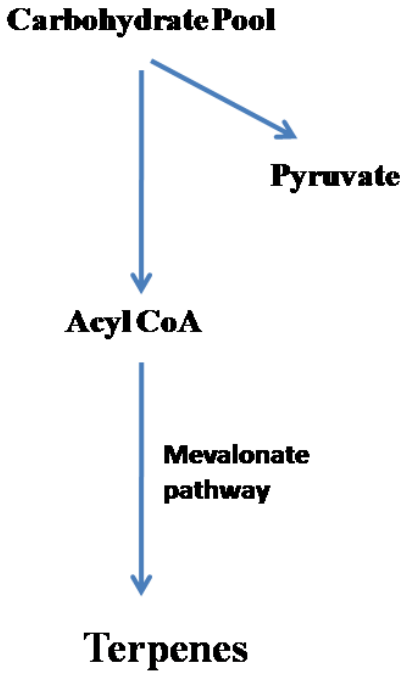


Fig 9 Biosynthesis of Terpenes

1.3.3.1.3 Aroma compounds derived from lipid metabolism

The majority of plant volatiles on a quantitative and qualitative basis originate from saturated and unsaturated fatty acids [80,81]. Fatty acid-derived straight-chain alcohols, aldehydes, ketones, acids, esters and lactones are found ubiquitously in the plant

kingdom at high concentrations, and are basically formed by three processes, α-oxidation, β-oxidation and the lipoxygenase pathway [80,81].

Many of the aliphatic esters, alcohols, acids, and carbonyls found in fruits are derived from the oxidative degradation of linoleic and linolenic acids. In addition, some of the volatile compounds derived from enzyme-catalyzed oxidative breakdown of unsaturated fatty acids may also be produce by autoxidation.

α and β oxidation of lipids

Although the degradation of straight chain fatty acids by α and β-oxidation is a major process for the formation of flavor molecules in all organisms, the specific pathways in plants are not well understood. The fatty acid α-oxidation mechanism in plants involves free fatty acids (C12–C18) that are enzymatically degraded via one or two intermediates to C (n-1) long-chain fatty aldehydes and CO_2. A dual-function α-dioxygenase/peroxidase and NAD+ oxidoreductase catalyze the α-oxidation of fatty acids in plants.

β-oxidation results in successive removal of C2 units (acetyl CoA) from the parent fatty acid. Short- and medium-chain linear carboxylic acids that are formed by repeated β-oxidative cycles followed by the action of an acyl CoA hydrolase have been found in many essential oils isolated from different plant sources. Aliphatic acids up to C10 play a significant role in flavors due to their sharp, buttery and cheese-like odors. These compounds also act as substrates in the form of their acyl CoAs for biosynthesis of other flavors. Aliphatic short and medium-chain aldehydes and alcohols are emitted by various plant parts and are formed by enzymatic reduction of the parent acyl CoAs.

Another major group of fatty acid-derived flavor molecules are alkanolides, which have c-(4-) or d-(5-)-lactone structures. Sensory important lactones usually possess 8–12 carbon atoms and some are very potent flavor components for a variety of fruits.

Lipoxygenase pathway

Lipids contribute to vegetable aroma primarily by lipoxygenase pathway [77]. Unsaturated fatty acids, mainly linoleic and linolenic acid, liberated from chloroplast membrane lipids have been demonstrated to be the precursors in the formation of the green aroma compounds via lipoxygenase pathway. The products of this pathway are collectively called green leafy volatiles (GLVs) as they impart the characteristic fresh green aroma to vegetables. GLVs are a group of eight compounds comprising of volatile aliphatic C6 aldehydes and alcohols and their corresponding hexyl esters as shown in Fig 10. Quantitative changes in these volatile constituents produce a green odor distinctive of the plant species.

Lipoxygenase pathway of GLV formation can be divided into following steps [77]:

1) Release of fatty acids from membrane lipids viz galactolipids, phospholipids and triglycerides via lipolytic acyl hydrolases or lipases.

2) Oxygenation of the released linolenic acid and linoleic acid to form fatty acid hydroperoxides by enzyme lipoxygenase.

3) Lysis of the fatty acid hydroperoxides \ between C-12 and C-13 by fatty acid hydroperoxide lyase (HPL) to form C6 aldehyde.

4) Conversion of the formed aldehydes to respective alcohols and esters by alcohol dehydrogenase (ADH) and alcohol acyl CoA transferase (AAT).

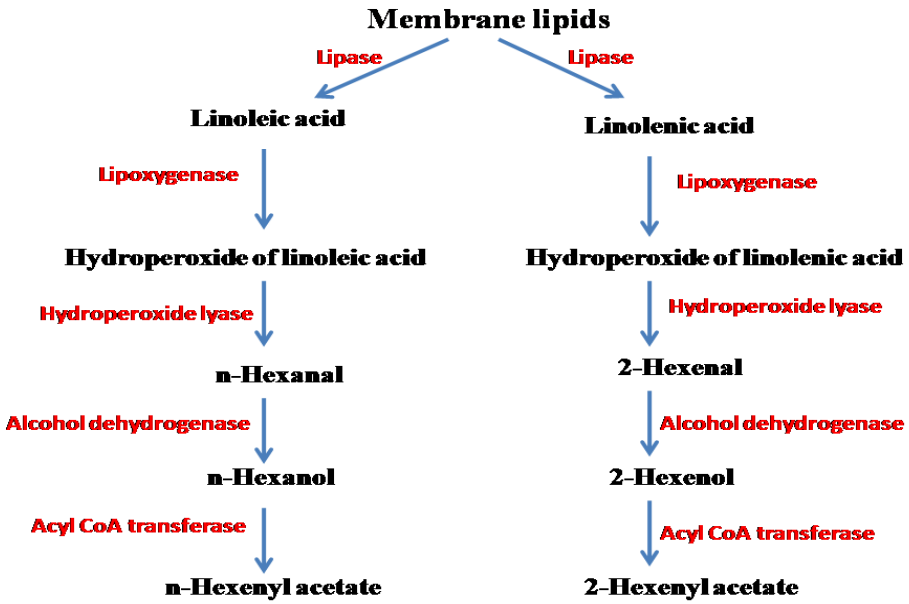


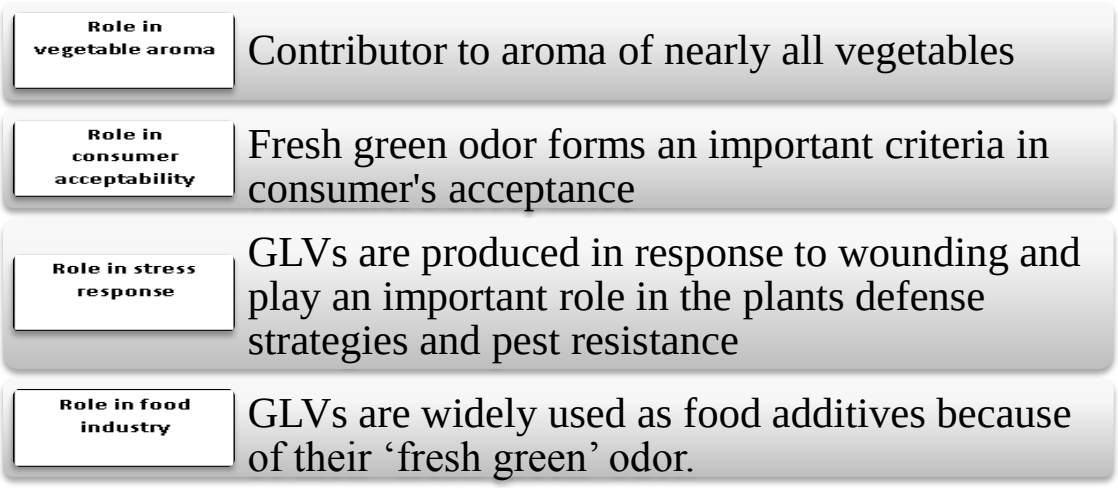
Fig 10 Lipoxygenase pathway of GLV synthesis

The first step of the LOX pathway i.e. the release of fatty acids from membrane lipids is considered to be the rate limiting step in GLV synthesis. Polar glyceroglycolipids are the major membrane lipids in photosynthetic organisms. Monogalactosyldiacyl glycerol (MGDG), digalactosyldiacylglycerol (DGDG) and sulfoquinvosyldiacylglycerol (SQDG) account for 90% of the total lipids in the chloroplast thylakoid membrane. Phospholipids and triglycerides account for rest of the lipid species. Lipases like phospholipases and galactolipases are known to be induced by stress like wounding and pathogen attack and act on respective lipid to release fatty acids. However, which lipases are of prime importance remains questionable. While some studies have shown galactolipases to be specifically involved in such response other authors have emphasised on the role of phospholipases.

LOX is a non-heme, iron-containing dioxygenase that catalyzes the regio- and enantio-selective dioxygenation of unsaturated fatty acids (e.g. linoleic and linolenic acid) containing one or more 1Z,4Z-pentadienoic moieties. Two types of LOX exists in nature,; one oxygenates specifically at C-13 (13-LOX) of linoleic and linolenic acid and other at C-9 (9-LOX) forming (13S)- and (9S)-hydroperoxy derivatives, respectively [77].

HPL cleaves the LOX products, resulting in the formation of oxo acids and volatile C6 and C9 aldehydes. Similar to LOX, HPL can be classified into two groups according to substrate specificity into 13-HPL and 9-HPL [77]. HPL is a member of the cytochrome P450 family CYP74B/C, and acts on a hydroperoxy functionality in a lipid peroxide without any co-factor. Lysis by 13-HPL generates C-6 compounds. Linoleic acid produces hexanal while linolenic acid produces 3-hexenal. These are further transformed to respective alcohols and esters by ADH and AAT respectively. 9-HPL forms nonanal which are responsible for characteristic aroma of cucumber.

Importance of lipoxygenase pathway



Byproducts of LOX pathway

Oxidative metabolism of PUFA give rise to a group of biologically active compounds, collectively termed as oxylipins [82]. The hydroperoxides derived from PUFAs are subsequently acted upon by different cascade of enzymes to form the oxylipins. These compounds play varied role in plants and are often correlated with plant defense response.

The HPL branch of lipoxygenase pathway directs the formation of C6 aldehydes and C12 ω-keto fatty acids. The C6 compounds are the GLVs associated with vegetable aroma as discussed earlier. The C12 product derived from linolenic acid is the precursor of traumatin, mitogenic compound that is implicated in wound healing.

Metabolism of 13-HPOT by DES produces divinyl ether fatty acids such as etherolenic acids. This metabolic route has been demonstrated in both green and non green tissues however its biological function remains yet to be elucidated.

Another fate for fatty acid hydroperoxides is reduction into their corresponding alcohols and further transformation by a peroxygenase (POX) to form epoxyhydroxy-PUFAs. These compounds being cutin monomers (structural component of cuticule) are believed to be involved in the response of plants to aggressions and also act as natural pesticides.

The allene oxide synthase (AOS) branch of LOX transforms 13-HPOT of linolenic aid to jasmonates [83]. Jasmonates represent the biologically active products of the AOS branch of jasmonic acid biosynthesis, which includes jasmonic acid as well as its derivatives like methyl jasmonate. The release of linolenic acid from chloroplast lipids represent the key regulatory step in wound-induced JA signaling. Their role as

signalling molecule is well established. The Jasmonate signal pathway involves several signal transduction events: 1) the perception of the primary stress stimulus and transduction of the signal locally and systemically; 2) the perception of this signal and induction of JA biosynthesis; 3) the perception of JA and induction of responses; and finally, 4) integration of JA signaling with outputs from the SA, ethylene, and other signaling pathways.

ACTION OF JASMONATES

- ❖ JA induce proteinase inhibitor proteins in response to wounding and pathogen attack playing role in plant defense.
- ❖ Regulates developmental processes including fruit ripening, root growth and senescence.
- ❖ Jasmonates are known to induce biosynthesis of several secondary metabolites in different plants species including alkaloids, terpenoids, phenylpropanoids and glucosinolates.

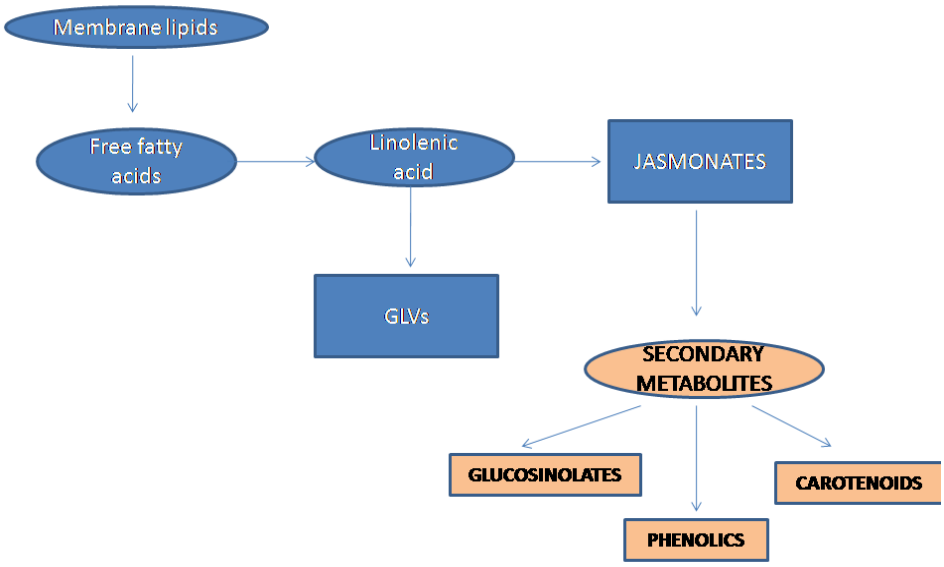


Fig 11 Role of lipid metabolism (LOX pathway) in synthesis of secondary metabolites

1.3.2.3 Genes involved in aroma synthesis in Brassica vegetables

The lipoxygenase pathway for GLV synthesis and glucosinolate biosynthetic pathways leading to isothiocyanate generation are the major contributer to aroma profile of Brassica vegetables. The main genetic pathway of GLV and glucosinolate biosynthesis has been identified in Arabidopsis using genetic and biochemical approaches.

1.3.2.3.1 Genes involved in GLV biogenesis

GLV synthesis in brassica vegetables are reported to be under genetic control and is induced as a result of stress response. Lipases, lipoxygenase and hydroperoxide lyase are the three enzymes associated with GLV synthesis from membrane lipids.

Although the underlying regulatory mechanisms responsible for GLV formation are undoubtedly complex and difficult to generalize, recent evidence indicates that phospholipases (PLs) that release fatty-acid precursors from membrane lipids are key components of this regulation. *PLA2* and *PLDα* are the key genes involved in regulation of phospholipases[84].

Lipoxygenase enzyme is reportedly translated by *LOX* family of genes. As many as 23 *LOX* genes have been detected in cucumber genome [84]. These genes are divided into type 1 and type 2, representing 9-LOX and 13-LOX respectively. These genes have been reported to be expressed differentially during development. The regulation of *LOX* gene expressions by different effectors, such as jasmonates, and ethylene, and by different forms of stress, such as wounding, cold and salt stress, have been revealed in some plant species in recent decades.

HPL enzyme involved in lysis of hydroperoxides to GLVs are produced from *CYP74B* which belong to cytochrome P450 gene family. However, unlike other

cytochrome P450 gene products that are localoized in mitochondria or endoplasmic reticulum HPL enzyme is localized in chloroplast membrane [84]. Unlike other cytochrome P450 gene products HPL has a low affinity for carbon monoxide and do not require NADH dependent cyt 450 reductase for its activity.

1.3.2.3.2 Genes involved in glucosinolate biogenesis

Several enzymes and transcription factors have been identified to be involved in the GSL biosynthesis in the model plant, Arabidopsis, and in a few other Brassica crop species[85]. Six MYB factors have been found to be transcriptional regulators in the biosynthesis of glucosinolate in Arabidopsis. While MYB28, MYB29, and MYB76 specifically transactivate genes related to aliphatic glucosinolate biosynthetic pathway (MAM3, CYP79F1, and CYP83A1) [86]. MYB34, MYB51, and MYB122 are regulators of the indolic glucosinolate biosynthetic pathway (TSB1, CYP79B2, and CYP7. A correlation between aliphatic GSLs content and gene expression level of MYB28 has been demonstrated by in Chinese cabbage [87]. MYB promoters were also found to regulate expression of the genes under osmotic stresses in *A. Thaliana* [88]. Miao et al reported an increase in aliphatic GSL concentration due to glucose intake [89]. Up-regulation of MYB28 was found to be the major reason behind it. Infact, a decrease in aliphatic GSL was noted due to down-regulation of CYP83A1 and CYP79F1 in MYB28 mutants. This indicates that the expression of transcription factors can sense changes in the environment in a shorter time than the production of secondary metabolites.

The glucosinolates in cells are hydrolyzed by the enzyme myrosinase [24]. Hence, glucosinolate content depends on myrosinase activity. Several myrosinase genes from Sinapis alba, Brassica napus, and Arabidopsis thaliana have been isolated and

characterized indicating that myrosinases are encoded by a multigene family consisting of three subgroups [90]. Myrosinase in the Brassica family is encoded by a gene family, which consists of three subfamilies, namely, MA (Myr1), MB (Myr2), and MC [85]. Several myrosinase-associated proteins, such as epithiospecifier modifier 1 (ESM1), ESP, and MVP1, have been identified in Arabidopsis, which are mainly involved in the generation of diversified GSL metabolic products [85].

1.3.2.4 Factors affecting aroma profile of brassica vegetables

Vegetables are subjected to a variety of biotic and abiotic stresses both in pre and post harvest stages. In post harvest stage while insect infestation and microbial contamination are the major biotic stresses, post harvest processing operations tend to provide abiotic stress to vegetables. Under stress rate of photosynthesis is reduced and carbon fixation is predominantly invested to secondary metabolite production. An array of secondary metabolites are produced, many of which either directly or indirectly are often associated with the aroma profile of the species. Processing operations enhances a diverse array of enzymatic pathways which in many cases are associated with generation of volatiles.

Membranes are the main targets of degradative processes induced by stress. Membrane lipids primarily, MGDG has been reported to be the lipid species most sensitive to stress [91]. Consequently, the lipid derived pathways, particularly; the lipoxygenase pathway of GLV synthesis is known to be induced by stress. GLVs are known to be released almost immediately after wounding. An increase in GLVs was also seen with increasing temperature due to cell wall rupture [92]. Lipid peroxidation of cell wall lipids by ozone leads to emission of GLVs [92]. Similar results were also seen for UV

treatment [92]. As GLVs are known to impart ‘fresh like’ aroma to brassica vegetables, change in their content can affect the consumer acceptability of these products.

Glucosinolates and their hydrolytic products are frequently investigated for their role in plant defense system against insects, herbivores, and certain microbial pathogens in brassica plants. It has been reported that environmental factors, such as light, temperature, salinity, water, CO_2 and drought are known to effect glucosinolate levels in Brassica species [93]. UV radiation has been shown to effect secondary metabolism in plants. Microarray data have shown that the genes related to the biosynthesis of flavonoids, glucosinolates, and terpenoids were differently expressed after UV-B radiation [93]. Post-harvest storage conditions of Brassica vegetables are also known to influence GSL and related isothiocyanate content. However, the effect during storage can vary depending on the origin and variety of the vegetable as well as the processing history. Content of these compounds was found to decrease in vegetables such as broccoli, brussel sprouts, cauliflower, and green cabbage when stored in a domestic refrigerator (4 8"C) for 7 days unlike when stored at ambient temperature [94]. Storage of vegetables at very low temperature (85"C) can result in freeze thaw fracture of plant cells leading to significant loss of GSLs as a consequence of their conversion to isothiocyanates during thawing [94]. Since, the glucosinolate breakdown products are known to impart characteristic aroma to brassica vegetables, stress induced by processing operations can alter the aroma profile of these vegetables thus affecting consumer acceptability.

The levels of other sulfur compounds of brassica namely, methanethiol, dimethyl sulfide and dimethyl sulfide, tend to change in response to processing operations and storage conditions. Cooking has been shown to increase the content of methanethiol,

dimethyl sulfide (DMS) and dimethyl trisulfide (DMTS) in brassica vegetables [78]. The *S*-methylmethionine sulfonium salt has been suggested to be the precursor of DMS. Temperature rise during storage has been documented to produce DMS in raw broccoli [78]. Packaging environment has also been shown to influence sulfide aroma content in packaged vegetables during storage. Storage of broccoli in high CO_2 caused an increase in DMDS and DMTS concentration compared to fresh broccoli sample. The high CO_2 concentration induces cellular deterioration resulting in degradation of *S*-methyl-L-cysteine sulfoxide by cysteine sulfoxide lyase (C-S lyase), thereby increasing the development of DMDS and DMTS. Change in pH also influences dimethyl disulfide in brassica tissue. Increase in pH tend to enhance cysteine sulfoxide lyase activity in cells causing an increase in DMTS content.

Volatile profile of minimally processed vegetables also tends to change in relation to microbial activities during storage [32]. Production of fermentative metabolites including ethanol and acetaldehyde during storage has been associated with off odors in packaged minimally processed vegetables. 2-methyl-1-butanol, 3-methyl-1-butanol and 1-propanol are the other volatiles produced due to microbial contamination in vegetables during storage [32]. These compounds impart alcoholic odor to the product making it less acceptable to consumers.

1.3.4 Cabbage (Brassica oleracea var *capitata) and its importance*

Cabbage (*Brassica oleracea* var *capitata*) is a leafy green or purple vegetable of brassica family, grown as an annual vegetable crop worldwide. India (7,949,000 tonnes) is the second largest producer of cabbage after China (31,750,000 tonnes) [95]. Fresh leaves of cabbage are used for preparation of a wide variety of recipes including

delicacies like sauerkraut and kimchi. Cabbage is also widely marketed as a minimally processed product in the shredded form due to the associated convenience and fresh characteristics. It is known for its nutritional value including antioxidant and anticancer properties. It is a rich source of phenolics, glucosinolates, vitamins and minerals. Isothiocyanates are reported to be mainly responsible for the observed chemoprotective activity of these vegetables [96]. In vitro and in vivo studies have reported that isothiocyanates affect many steps of cancer development including modulation of phase I and II detoxification enzymes, functioning as a direct or as an indirect antioxidant by phase II enzyme induction, modulating cell signalling, induction of apoptosis, control of the cell cycle and reduction of heliobacter infections [96].

Owing to the popularity of this vegetable, many studies have been reported, concerning cabbage [76,97]. Composition of volatile constituents has been investigated to evaluate the effects of variations in cooking methods [98] and to compare the volatile fractions from various cabbage varieties [98]. Studies to improve the post harvest shelf life of cruciferous vegetables [98] have emphasized the need for an improved knowledge of the compounds contributing to their flavor. While radiation processing is a promising tool for enhancing the shelf life of this vegetable, very few reports have dealt with the changes in chemical constituents during such a treatment. However, extensive work is required to study the effect of gamma irradiation on phytochemical constituents of cabbage in order to understand the sensory and nutritional status of the radiation processed product.

1.4 Scope of the work: Aims and objective

Plants, when exposed to unfavorable environments, such as water deficit, chilling, heat stress, oxygen deficiency, and air pollution, result in varying degree of stress. Plants adapt to unfavorable conditions through genetically determined stress resistance. Unlike typically processed foods, fresh-cut products consist of living tissues and post harvest processing treatments including radiation processing can act as abiotic stress bringing about change in post harvest physiology of the product. There are few reports on the impact of radiation processing on the post harvest physiology of leafy green vegetables particularly that of Brassica species. Consumers and food safety advocates are, therefore, worried about the nutritional and chemical quality of such radiation treated produce. Even though radiation processing is considered an effective method of food preservation, public misconception about irradiation have delayed many of its potential application in the food industry.

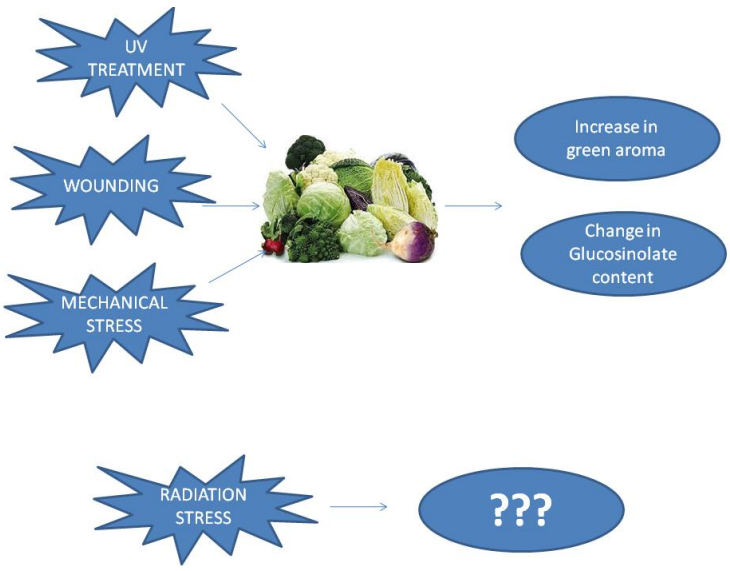


Fig 12 Effect of stress on aroma and glucosinolate profile of vegetables

Production of ROS to protect against abiotic and biotic stress is one of the major mechanisms by which plant protect it against adverse environmental conditions. However, emerging data show that ROS production in certain situation can also contribute to the physiology and increased fitness of plants. The effect of different postharvest abiotic stresses (*i.e.*, wounding, UV-light, hyperoxia, and the exogenous application of ethylene and methyl jasmonate) on the accumulation of phenolic compounds in fruits and vegetables has been evaluated in several studies. Nevertheless, little is known on the physiological and molecular basis for the accumulation of antioxidants as a postharvest stress response. Increasing the scientific knowledge in this area is critical to envisage strategies that permit the effective use of crops as biofactories of nutraceuticals. However, the physiological and molecular basis for this stress response remains unknown.

Genetic engineering is the most commonly used tool to generate crop lines with enhanced concentrations of desirable secondary metabolites. However, metabolic engineering is technically complex and the extensive cultivation of genetically modified crops has been questioned due to potential environmental and safety issues. Taking this into account, post harvest stress type treatments can be exploited as an effective alternative to genetic manipulation.

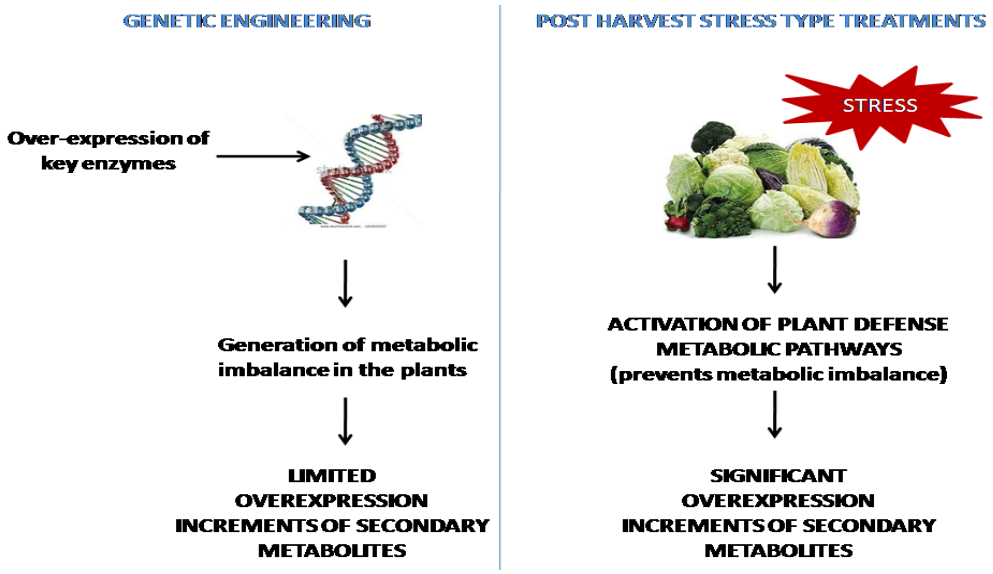


Fig 13 Comparison between genetic engineering and postharvest abiotic stresses as secondary metabolites overexpression strategies.

Membranes are the main targets of degradative processes induced by stress including radiation stress. ROS produced during water radiolysis tend to bring impairment in membrane integrity and structure. Effect of radiation processing on lipids mainly phospholipids and the consequent formation of off-odors in high fat containing foods have been extensively reported in literature. However, effect of radiation processing on lipid profile of Brassica vegetables is yet to be explored.

Low dose gamma irradiation has also been shown to induce phenylpropanoid metabolism in irradiated potatoes thereby enhancing phenolic synthesis. This demonstrates the potential use of abiotic stresses as a tool for the production of high commercial value plant bioactives. However, the physiological and molecular basis for this stress response remains unknown. Correlation between physiology, biochemistry and secondary metabolite induction by gamma irradiation is needed. Molecular knowledge of radiation stress response and tolerance mechanism can provide insight

into the how these are regulated and could be the basis for increasing nutritional and sensory quality. This can aid in demonstrating the role of radiation processing in enhancing quality and in alleviating fear among consumers about the safety of irradiated leafy green vegetables. The use of radiation stress on extensively grown crops for the production of secondary metabolites with health-promoting properties can be of scientific, economical and social interest.

The present thesis aims at understanding the effect of gamma irradiation on post harvest physiology of Brassica vegetables affecting the aroma profile of the vegetables. The mechanism of the changes is further studied on biochemical and molecular level.

The specific objectives of the thesis are detailed below:

- Isolation, identification and quantification of polar membrane lipids in selected Indian vegetables and determining their metabolic changes during radiation processing.
- Analysis of lipoxygenase and hydroperoxide lyase activities, consequently resulting in qualitative and quantitative changes in green odor compounds and impact on odor quality.
- Understanding the mechanism of radiation induced changes in aroma profile at molecular level.
- Use of radiation processing for development of minimally processed ready to eat shredded cabbage.

CHAPTER 2

MATERIALS AND METHODS

2.1 Plant Material

Vegetables of brassica family, namely, cabbage (*Brassica oleracea var capitata*) cauliflower (*Brassica oleracea var botrytis*), broccoli (*Brassica rapa)* and radish (*Raphanus sativus*) were procured from local market, Mumbai, Maharashtra. These samples were designated as market samples.

Cabbage samples of BC-79 and NS-22 varieties were also obtained from farmers of Akola district, Maharashtra, India. The samples were authenticated at Dr.Panjabrao Deshmukh Krishi Vidyapeeth, Akola as belonging to the above varieties. Harvesting was done 65 days after planting when the vegetable was known to be mature.

2.2 Irradiation of samples

Cabbage samples were subjected to gamma irradiation using a ^{60}Co gamma irradiator (GC-5000, BRIT, India, dose rate 3.4 kGy/h, DUR 1.12) in air to an average absorbed dose of 0.5, 1, 2 and 2.5 kGy. Irradiation was carried out at room temperature. Dosimetry was carried out using a Fricke dosimeter before the start of experiment. Uncertainty of the doses was found to be within the limit of ±1 %.

2.3 Isolation, identification and quantification of aroma compounds

Isolation of volatile aroma compounds from 4 different brassica vegetables namely, cabbage, cauliflower, radish and broccoli were carried out by solid phase microextraction (SPME). Volatile oils from different cabbage varieties were also isolated by steam distillation using Likens-Nickeron simultaneous distillation apparatus.

2.3.1 Isolation of aroma compounds by SPME

a) Sample preparation: 100 g of samples (cabbage, cauliflower, broccoli and radish) were homogenized with 10 mL water. The homogenate was filtered through Whatman filter 1 and centrifuged at 12000 rpm for 20 min. 15 mL of supernatant was taken in a 40 mL SPME vial to which 4.5 g NaCl was added for inhibition of enzymatic activities and release of volatiles. 2-octanol (20 ug) was also added as an internal standard to the vials.

b) Headspace volatile analysis: Volatile compounds were extracted using SPME fiber (Supelco, Bellefonte, PA) having a coating of 50/30 μm polydimethylsiloxane (PDMS)/ carboxen (CAR)/ divinylbenzene (DVB). SPME extraction conditions such as temperature and time for equilibration and extraction of headspace volatiles were optimized. Optimum conditions for extraction of volatiles were equilibration at 37 °C for 45 min followed by extraction using SPME fiber for 20 min. After equilibration, volatile extraction was carried out by inserting a pre-conditioned (270 °C, 5 min) PDMS/DVB/CAR fiber through a septum of SPME vial. The fiber was exposed in sample headspace for 20 min. The SPME device was finally inserted into GC-MS injection port kept at 270°C and the fiber was exposed for 5 min.

c) GC/MS analysis: Analysis was carried out on GC/MS (QP5050, Shimadzu Corporation, Japan) equipped with a DB5 capillary column (length = 30 m, inner diameter = 0.25 mm, film thickness =0.25 μm, Restek Corporation, USA). Injections were conducted in splitless mode (5 min) and GC temperature settings were: 40 to 200°C at the rate of 4°C/min, held at initial temperature and at 200°C for 2 min. and further to 280°C at the rate of 10°C/min, held at final temperature for 10 min. MS parameters were: ionization voltage 70 eV, electron multiplier voltage, 1 kV and scan mode from m/z 40 to 350. Peaks were identified by comparing their mass fragmentation pattern (Wiley/NIST Libraries), retention time and Kovats index with standards. The amount of each individual compound present in the sample was calculated by mean of the

internal standard ($mass_{unknown}$ = ($mass_{IS}$/$area_{IS}$) x $area_{unknown}$), and expressed as mg per kg of dry weight.

2.3.2 Isolation of aroma compounds by SDE

a) Extraction: 200 g of cabbage leaves were blended with 200 ml of chilled water containing 1.5% NaCl. 500 µg eugenol was added as internal standard. The mixture was subjected to steam distillation using Nikersons-Likens simultaneous distillation extraction apparatus. Extraction was carried out with ether (80 mL) as extracting solvent. The organic layer containing the volatile constituents was concentrated to a volume of 12 ml using Kuderna Danish apparatus. Further concentration using a slow stream of nitrogen resulted in volatile oil free of solvent. The volatile oils (mg/wet weight) thus obtained were then subjected to GC-MS analysis.

b) GC/MS analysis: Cabbage essential oil obtained from steam distillation were subjected to GC–MS analysis on a Shimadzu GC–MS instrument (Shimadzu Corporation, Kyoto, Japan) using the parameters described above.

2.4 Isolation, identification and quantification of cabbage lipids

2.4.1 Isolation, identification and quantification of lipid species

a) Extraction: 300 g of cabbage leaves were ground in liquid nitrogen and soaked in 900 mL of chloroform: methanol (2:1) solution under an atmosphere of nitrogen to minimize oxidation on storage. The extract thus obtained was filtered and the residue soaked again in 500 mL of chloroform: methanol (2:1) solution. The process was repeated till a pale yellow filtrate was obtained. The subsequent filtrates were pooled together and centrifuged at 12000 rpm for 15 min. The supernatant thus obtained was concentrated in rota evaporator (Buchi Rotavapor-114) to make 1 % solution which was used for subsequent assays.

b) TLC analysis: The total lipid extract thus obtained was subjected to silica gel TLC (Kieselgel 60, Merck, Germany). Neutral lipids were analyzed using solvent mixture of hexane: diethyl ether: acetic acid (80:20:2) while phospholipids were separated identified using ethyl acetate: 2-propanol: chloroform: methanol: 0.25% aq KCl (25:25:25:10:9) as the developing solvent system. Separation of galactolipids was carried out using chloroform: methanol: water (80: 18: 2) as the solvent system. The individual lipid class was identified from Rf values of standards spotted separately on the same plate. The separated spots were visualized by exposing to iodine vapor and the area of the individual spots was quantified on a TLC-densitometer (CS9301PC, Shimadzu, Japan) from a standard curve of spot area vs. concentration using different concentrations of standard lipid species referred above.

2.4.2 Isolation, identification and quantification of total fatty acids

50 mg of chloroform: methanol extract was saponified with 2N KOH for 2 hrs at 90 ºC acidified with 6N HCl and then extracted was done with diethyl ether (3 x 30 mL). The ether extract was washed free of acid and dried over anhydrous sodium sulfate. 50 µg of dodecanoic acid was added as internal standard. The extract was evaporated to dryness in rota vapour. The residue containing free fatty acids was converted to their methyl esters by treating with diazomethane (30 mL) overnight. Diazomethane was removed completely in a slow stream of nitrogen and the resultant residue was dissolved in chloroform to make 1% solution. Further analysis was done by GC/MS.

a) GC/MS analysis: The operating conditions were: column temperature programmed from 140 to 200°C at the rate of 4°C/min, held at initial temperature for 5 minutes and at 200°C for 7 min and further to 280°C at the rate of 10°C/min, held at final temperature for 15 min. Other parameters were identical to that for the analysis of volatile oil. Peaks were identified by

comparing their mass fragmentation pattern with standard spectra available in the spectral library (Wiley/NIST Libraries) of the instrument as well as by comparing their retention time and Kovats index with available standards. The amount of each individual compound present in the sample was calculated by mean of the internal standard, and expressed as mg per kg of dry weight.

2.4.3 Analysis of fatty acid composition of MGDG and TAG

To analyze the fatty acid composition of MGDG and TAG, total lipid extracts were subjected to preparative (0.5 mm thickness) silica gel TLC using solvent system employed for neutral lipid and galactolipid separation as described in section 2.4.1. Around 30 plates were developed for both the lipid species. The bands corresponding to TAG and MGDG from different plates were scraped out and soaked in chloroform: methanol (2:1) overnight. The extract was filtered with Whatman filter 1, hydrolyzed with 2N HCl for 3 hours. The compounds of interest were partitioned into diethyl ether and then converted methyl derivative with diazomethane as discussed in section 2.4.2. The methylated fatty acids derived were analyzed on a GC/MS using same parameters as for fatty acid analysis.

2.5 Isolation, identification and quantification of cabbage glucosinolates

a) Extraction: 5 gm of freeze dried cabbage samples were extracted with 100 ml of boiling water containing glucotropaeolin (100 µl, 20 mM) as internal standard for 10 min. The slurry was then filtered and the residue was re-extracted with boiling water (10min). The filtrate obtained after subsequent workup was pooled with the main extract. The crude aquous extract was re-extracted successively with ethyl acetate (3 x 30 mL) & then with water saturated n-butanol (3 x 30 mL). The n-butanol and ethyl acetate extract was dried over sodium sulfate concentrated to dryness in rota-evaporator and the residue made to a 10% solution in distilled water.

b) TLC analysis: The different extracts as obtained above were subjected to silicagel TLC using n propanol: ethyl acetate: water (7:1:2) as the developing solvent system. The separated spots were visualized by exposing to iodine vapor and the Rf of the individual spots were noted. Glucosinolates were identified by comparing their Rf values with standard compounds and with literature value. As n-butanol extract was mainly composed of glucosinolates this extract was chosen for further studies.

c) HPLC analysis: The n-butanol extract was subjected to HPLC (Jasco HPLC system, Japan). Samples were eluted from a reverse pase C18 column (250 mm x 4.6 mm, 10 µ; HYPERSIL, Chromato-pack, Mumbai, India) using 50 mM ammonium acetate as solvent A and 50 mM ammonium acetate: methanol (80:20) as solvent B, under a solvent gradient of time 0 min, A= 100%; time 40 min, A= 0% at a flow rate of 0.3 mL/min. Wavelength was set at 235 nm. Sinigrin, the major glucosinolate in cabbage, was identified by comparing its Rt with that of the standard compound. Glucosinolates were desulfated using 10 mL crude aqueous extract (10% solution) to which 500 µl of 0.02 M sulfatase enzyme in aq. NaAcO-AcOH (pH 5) was added and incubated overnight. The resultant mixture was subjected to HPLC analysis as above for further confirmation of glucosinolates.

d) LC/MS Analysis: The n-butanol extract was subjected to LC/MS analysis. Mass spectra were recorded by atmospheric pressure chemical ionization in the negative mode using a Varian Ion Trap MS (410 Prostar Binary LC with 500 MS IT PDA detectors) equipped with a C-18 reverse phase stainless steel column (30 cm × 0.46 cm). All samples were filtered through a 0.45 µm filter (Millipore Corp.) before injection. The capillary voltage was kept at 80 V, and the air (nebulizing gas) pressure was 35 psi. Full scan data acquisition was performed by scanning from m/z 100 to 900.

2.6 Isolation, identification and quantification of phenolic compounds of cabbage

a) Extraction: Forty grams of cabbage were extracted twice in 150 mL of aquous methanol. The extract was filtered (whatman filter 1) and centrifuged at 12000 rpm for 20 min. The supernatant was concentrated in rota evaporator to make a 1 % solution.

b) TLC analysis of phenolic compounds: The 1% solution of different cabbage extracts as obtained above was subjected to silica gel TLC using toulene:etanol:formic acid () as developing system. The individual phenolic compounds were identified from the Rf values of standards spotted separately on the same plate. The separated spots were visualized by exposing to iodine vapor and the area of the individual spots was quantified on a TLC-densitometer (CS9301PC, Shimadzu, Japan) from a standard curve of spot area vs. concentration using different concentrations of standard phenolic compound.

c) HPLC analysis of phenolic compounds: The methanol extract was subjected to HPLC (Jasco HPLC system, Japan). Samples were eluted from the column using 0.1% formic acid as solvent A and methanol as solvent B. Solvent gradient employed were time 0 min, A= 80%; time 35 min, A= 50%; time 37 min, A= 20% at a flow rate of 1 mL/min. Wavelength was set at 280 nm. Phenolics were identified by comparing their Rt with standard compounds and from literature values. Salicylic acid (100 µl, 20 mM) was used as internal standard. Peak quantification was achieved by a calibration curves obtained for each reference standard. Co chromatography with authentic standards was also performed for further confirmation of the identified phenolic constituents.

2.7 Analysis of enzyme activities

2.7.1 Lipase assay

a) Extraction: Extraction was done according to Pérez et al [99]. Briefly, 20 g of cabbage leaves were extracted in 60 mL of ice cold extraction buffer (0.1M TrisHCl, pH 8) containing 0.1M KCl, 0.1% Triton X-100 and 2g PVPP. The resulting suspension was centrifuged at 14000 rpm at 4°C for 20 min, and the supernatant was used for lipase activity determination.

b) Assay: Lipase activity was measured spectrophotometrically as described earlier with some modifications [99]. Reaction was started by the addition of 1mL enzyme extract to 2.5 mL p-nitrophenyl laurate [420 µM] in 2.5 mL TrisHCl buffer (0.1 M, pH 8.2). Absorbance was monitored on a spectrophotometer (UV-2450, Shimadzu, Japan) up to 15 min. p-Nitrophenol standard curve was used to convert absorbance to µM substrate hydrolyzed.

c) Preparation of standard curve: One ml of p-nitrophenol blue was mixed with 49 ml of tris HCl buffer (pH 8). Different concentrations (0.05, 0.1, 0.2, 0.4, 0.5 ml) of p-nitrophenol blue solution as obtained above were taken in different test tubes and final volume made to 5 ml with Tris Cl buffer. Tris HCl buffer pH 8 was taken as blank. Absorbance was measured at 410 nm.

2.7.2 Lipoxygenase assay

a) Enzyme extraction: Cabbage leaves (20 g) were extracted in 60 mL of ice cold extraction buffer according to Gardner [100]. 20 g of cabbage leaves were ground in liquid nitrogen, stirred and homogenised for 2 min on ice in 100 ml of sodium phosphate buffer (0.1 M, pH 7). The resulting suspension was centrifuged at 14000 rpm at 4°C for 20 min, and the supernatant was used for LOX activity determination.

b) Preparation of linolenic acid substrate: Linoleic acid substrate was prepared according to the procedure followed by Gardner [100]. To a mixture of tween 20 (0.5 ml) and borate buffer (10 ml) linoleic acid (0.5 mL) was added dropwise with vigorous vortexing to get a fine emulsion.To this emulsion 1N NaOH (2.3 ml) was added and the final volume made to 200 ml with water.

c) Assay: LOX activity was measured spectrophotometrically at 234 nm by the method reported by Gardner [100]. Volume of enzyme extract and linoleic acid substrate in the reaction mixture was optimized. The reaction mixture (3.0 ml) contained linoleic acid substrate (10 µl), acetate buffer (0.1 M, pH 5) and extract (30ul). Absorbance was measured for 10 min using a spectrophotometer. An extinction coefficient of 25000 $M^{-1}cm^{-1}$ was used to convert absorbance values at 234 nm to µmol of conjugated diene.

2.7.3 Hydroperoxide lyase assay

a) Enzyme extraction: Extraction procedure followed was same as for lipase assay as discussed in section 2.7.1.

b) Assay: HPL activity was assayed by the loss in absorption at 234 nm by the hydroperoxide as described previously with slight modifications [101]. Briefly, linoleic acid substrate (0.6 mL of 7.5mM) was incubated with 1.12 mg of soybean lipoxygenase (100000 units/mg) in 30 mL distilled water for 1 hr to obtain a solution of hydroperoxide substrate. The final reaction mixture contained 250 µl of the hydroperoxide substrate prepared earlier and 250 µl of enzyme solution made up to a volume of 3 mL with potassium phosphate buffer (0.1 M, pH 6). Readings were taken for 10 min by a spectrophotometer (UV-2450, Shimadzu, Japan). An extinction coefficient of 25000 $M^{-1}cm^{-1}$ was used to convert absorbance values at 234 nm to µmol of products formed.

2.7.4 Myrosinase assay

a) Extraction: 40 g of cabbage leaves were blended in a homogenizer with 100 mL cold sodium phosphate buffer (33 mM, pH 7) containing 0.2M NaCl. The resulting suspension was centrifuged at 14000 rpm at 4 °C for 20 min and the supernatant was used for myrosinase activity determination.

b) Assay: Assay was carried out according to the method proposed by Shapiro et al [102]. Briefly, 0.2 mM sinigrin, 500 μM ascorbic acid and 1 mM EDTA were incubated at 37 °C for 5 min. Reaction was started by addition of 50 μl of supernatant. Readings were taken for 10 min by a spectrophotometer (UV-2450, Shimadzu, Japan). An extinction coefficient of 6780 M^{-1} cm^{-1} was used to convert absorbance values at 235 nm to μmol of products formed.

2.7.5 Phenylalanine ammonia lyase (PAL) assay

a) Extraction: PAL activity was measured according to the protocol proposed by Degl'Innocenti et al with some modifications [103]. Sample (10 g) was homogenized with 30 mL of cold borate buffer (50 mM, pH 8.5) containing 10 mM 2-mercaptoethanol and 0.2 g of PVPP. The homogenate was filtrated through 4 layers of cheesecloth and centrifuged at 12,000 rpm at 4 °C for 20 min. The supernatant was used for subsequent assay.

b) Assay: PAL activity was measured after the addition of 2 mL of 50 mM l-phenylalanine to 1 mL of the supernatant and incubation at 40 °C for 1 h. The absorbance was measured at 290 nm before and after incubation. Difference between the two gave the amount of product (cinnamic acid) formed. One unit of PAL activity equals the amount of PAL that produced 1 μmol of *trans*cinnamic acid in 1 h and was expressed as μmol g^{-1} FW h^{-1}.

2.7.6 Polyphenoloxidase (PPO) assay

a) Extraction: Extraction was done according to the procedure reported by eaerlier [103]. Tissue (10 g) was homogenized at 4 °C with 30 mL of 50 mM phosphate buffer (pH 6.5). The homogenate was filtered and centrifuged at 12000 rpm at 4 °C for 20 min. The supernatant was used for subsequent assays.

b) Assay: The standard reaction mixture consisted of 250 µL of 0.2 M sodium phosphate buffer (pH 6.0), 50 µL of 1.0 M catechol, and 50 µL of enzyme solution. The reaction was carried out at 30 °C for 5 min, and PPO activity was measured by monitoring the increase in absorbance at 420 nm. One unit of PPO activity was defined as the amount of enzyme that proposed resulted in an increase in absorbance of 0.01 per minute.

2.7.7. Peroxidase (POD) assay

a) Extraction: Extraction procedure followed was same as that for PPO assay (section 2.7.6).

Assay: Assay was carried according to the procedure reported by Degl'Innocenti et al (2005) with some modifications[103]. The chlorogenic acid peroxidase assay contained 800 µL of 50 mM potassium phosphate buffer, pH 6.5, 50 µL of 80 mM chlorogenic acid, 50 µL of extract, and 100 µL of 35 mM H_2O_2. The caffeic acid peroxidase assay contained 800 µL of McIlvaine buffer (114 mM Na2HPO4 and 43 mM citric acid), pH 5.5, 50 µL of 80 mM caffeic acid, 50 µL of extract, and 100 µL of 35 mM H_2O_2. In all cases, POD assays were initiated by the addition of H_2O_2 (100 µL, 35 mM). Absorbance was measured at 410 nm for chlorogenic acid and 470 nm for caffeic acid peroxidase activity. The activities of PODs were expressed as A_λ min^{-1} g^{-1} fresh weight.

2.8 Sample preparation for shelf life extension studies

a) Sample Preparation: Fresh market samples of cabbage were washed manually with tap water, any bruised or damaged items were removed and only healthy fresh vegetables were selected. Samples were cut with sterile stainless steel knives into strips (1 cm wide by 3.0 to 3.5-cm). Cut samples were packed (40 g) into polystyrene trays (inner dimensions: 9 cm × 9 cm × 2.5 cm). The trays were then over-wrapped all around with cling film.

Another set of cut samples were also stored in an atmosphere of volatile compounds For this purpose, different concentrations of volatiles were spotted onto a piece of filter paper which were subsequently placed inside the trays just before wrapping with cling film. The different volatiles used were allyl isothiocyanate, *trans*-hex-2-enal, *cis*-hex-3-enol, hexenyl acetate. The concentrations spotted were 0.005 µL/ml, 0.01 µL/ml, 0.05 µL/ml, 0.1 µL/ml (volume of volatile/ volume of tray).

b) Irradiation and storage: Packaged samples were subjected to various radiation doses (0.5, 1.0, 1.5, 2.0 and 2.5 kGy) as described in section 2.2. Irradiated samples were stored in the dark at 4 ± 1 °C and 10 ± 1 °C. In the present study, non-irradiated samples were acted as control samples during the entire storage period. Three replicates were prepared for each dose and storage day. The samples were examined on 0, 5, 8, 13, 16 and 21 days after packaging.

2.9 Microbial analysis

Standard methods were used to enumerate microorganisms present in minimally processed cabbage at each sampling time [104]. Mesophilic bacteria, yeast, and mold counts were carried out in triplicate for each single tray using Plate Count Agar and the pour plate method [104]. Cabbage sample (25 g) from each tray were cut with a sterilized knife in laminar and taken in

stomacher bag containing 225 mL sterile physiological saline. The sample was homogenized in a stomacher instrument at 260 rpm for 1 min. After appropriate serial dilutions, the samples were pour plated on plate count agar (PCA). The colonies were counted after 24 h of incubation at 37°C.

Total yeast and mold counts were performed with the pour plate method using potato dextrose agar supplemented with 0.1% tartaric acid to maintain pH of the media at 3.5. Plates were incubated at 37°C for 48 h. Microbial counts were expressed as $\log_{10}$ CFU g^{-1} of vegetable. Each analysis was performed in triplicate.

2.10 Sensory analysis

Sensory analysis was carried ou for cabbage and other vegetables usin the protocols described below:

2.10.1 Sensory analysis of irradiated brassica vegetables for preliminary screening

a) Sample preparation: Irradiation (0.5kGy, 1 kGy, 2 kGy and 2.5 kGy) were cut into strips (1 cm wide by 3.0 to 3.5-cm). For cauliflower and broccoli individual florets were cut from the stalk while radish samples were cut into discs. Untreated fresh cut samples served as control.

b) Sensory analysis: Sensory analysis was carried out by quantitative descriptive analysis (QDA) [43] at all doses by a sensory panel of 15 members (7 women and 8 men). The trained panelists were chosen according to following criteria: people with no food allergies, nonsmokers, with age between 25 and 55 y and performance on preliminary screening tests. The sample replicates were assessed in three different sessions to avoid tiredness and saturation. The panelists were asked to assess the whole sample and rinse their mouth with water in between. The panel analyzed the sample on an unstructured scale of 15mm. The scores given for all the parameters

for each sample were tabulated. Next, the mean value was calculated for each attribute, representing the panel's opinion about the sensory quality of the product and significant difference was found by ANOVA.

2.10.2 Sensory analysis of minimally processed cabbage at different storage points

a) Sample preparation: Cooked samples (control and irradiated**)** were analyzed by the panelist in different sessions. The cooked samples were prepared by boiling RTC cabbage in water for 5 minutes and immediately cooling it in chilled water. Boiling time was chosen in preliminary experiments by serving samples boiled for different duration (2 min, 5 min and 10 min) to the panelist. Amongst these the samples boiled for 5 min were liked most. Cabbage samples (10 g) were served in white trays numbered randomly to the sensory panel.

b) Sensory analysis: Sensory analysis was carried out by hedonic test at all doses by a sensory panel of 15 members (7 women and 8 men). All panelists had previous experience in carrying out sensory analysis of similar food products. Hedonic test was carried out using a 9- point scale with 1, dislike extremely or not characteristic of the product and 9, like extremely or very characteristic of the product [44]. Parameters evaluated were color, aroma, texture, taste and overall acceptability. To determine the acceptability of the samples at different storage points, all the parameters analyzed were compared with fresh control samples on each day. The scores given for all the attributes for each sample were tabulated. Next, the mean value was calculated for each attribute of a sample, representing the panel's judgment about the sensory quality of the product and significant difference was found by analysis of variance (ANOVA).

2.10.3 Evaluation of browning intensity

Evaluation of browning at the cut edges of cabbage strips were scored visually. A sensory panel of 15 members (6 men and 9 women) analyzed the samples. The rating scale reported by Ke and

Saltveit [105] was used to estimate visually the extent of browning in cut edges: the score ranged from 0 (no browning) to 9 (complete browning of cut).

2.10.4 Determination of colour

Color of the cabbage strips were measured by a colorimeter. Nine strips of cabbage were selected randomly from each packaged tray at different storage period for 21 days. Colour of the samples were measured by Minolta Chromameter (model CM-3600d Konica Minolta Sensing Inc., Japan). Instrument calibration was done with a white tile supplied with it and then used to determine the color using the 3 Commission Internationale de l'Eclairage (CIE) coordinates, L (lightness), a (−green, +red), and b (−blue, +yellow). Since the major change in the sample was due to darkening at the cut edges which was represented by the L values, the change in colour of the samples during storage was analyzed by monitoring the L values at each storage point.

2.11 Texture analysis

The texture analysis for the sample was performed using a Texture Analyzer (TA. HD. Plus, Stable Micro Systems). Nine strips of cabbage were selected randomly from each packaged tray at different storage period upto 21 days. The puncture resistance of the strips represents the overall texture of the product. Puncture strength of the strips (1 cm × 3 cm) were determined by 2 mm needle probe having test speed of 30 mm/min.

2.12 Analysis of head space gas composition

O_2 and CO_2 content was analyzed at 2 kGy using a gas chromatograph (GC 2010, Shimadzu Corporation, Japan). The GC was equipped with split/splitless injector, a molecular sieve column (length 30 m, 0.35 I.D., RT-Msieve 5A, Restek Corporation, USA) and a TCD detector. Injection port temperature was 35 $^{\circ}$C. Initial column temperature was kept 30 ◦C for 5 min and then raised

at rate of 0.167 °C/s to 100 °C. The column was further held at 100 °C for 5min with the TCD current and temperature maintained at 90mA and 110 °C, respectively. Sampling was done by inserting a hypodermic needle into the bag through an adhesive septum, previously stuck to the bag. A 0.1mL of headspace sample was extracted and injected into the GC at a split ratio of 5. Only O_2 and N_2 could be evaluated on the column used in the study. Based on observed O_2 and N_2 concentrations in the package headspace, actual concentrations of O_2 and CO_2 ($\%O_2$ and $\%CO_2$) were calculated using following equations:

$$\% O_2 = (\text{Observed } \%O_2 / \text{Observed } \%N_2) \times 78.084$$

$$\%CO_2 = 100 - [(\text{Observed}\%O_2 / \text{Observed}\%N_2) \times 78.084 + 78.084]$$

(atmospheric composition of N_2 taken as 78.084%)

2.13 Analysis of nutritional quality

a) Extraction: Forty grams of cabbage were extracted twice in 150 mL of aquous methanol. The extract was filtered (whatman filter 1) and centrifuged at 12000 rpm for 20 min. The supernatant was concentrated in rota evaporator to make 1 % solution.

2.13.1 Total phenolic content

Total phenolic content was evaluated in accordance with the Folin–Ciocalteu procedure [106]. Part of the methanolic extract obtained as above was treated with poly vinyl poly pyrrolidone (10 g/L, PVPP, Sigma–Aldrich, USA) to remove phenolic compounds from the extract. The mixture was then incubated overnight in an orbital shaker at 25 °C (2.5 oscillations per s). PVPP was then removed by centrifugation at 12000 rpm for 10 min at 4 °C. The supernatant was collected and the sediment (PVPP–polyphenol complex) was discarded. The absorption of the supernatant and original extract was measured at 725 nm using UV–visible spectrometer in accordance with the

Folin–Ciocalteu procedure. The content of total phenolics in cabbage was determined by the difference between phenolic content obtained before and after PVPP treatment and then expressed as the Gallic acid equivalent mass per kg of cabbage as mg kg^{-1}.

2.13.2 Total Flavanoid Content

The $AlCl_3$ method reported by Luximon-Ramma et al. was used for determination of total flavonoid content[107]. Aliquots of 1.5 mL of extracts were added to equal volumes of a solution of 2% $AlCl3.6H_2O$ (2 g in 100 mL methanol). The mixture was vigorously shaken, and absorbance was read at 367.5 nm after 10 min of incubation. Flavonoid content was expressed as µg Quercitin equivalent (QE) g^{-1} of cabbage.

2.13.3 DPPH assay

A DPPH radical scavenging assay was used to evaluate total antioxidant activity of cabbage [107]. An aliquot of methanolic extract (100 µL) was added to 1 mL of DPPH solution (110 µM in 80% aq methanol). After incubation under dark for 20 min absorbance was measured at 516 nm. Total antioxidant activity was expressed as µg Gallic acid equivalent (GAE) g^{-1} of cabbage.

2.13.4 Ferric reducing power assay

The Fe^{3+} reducing power of the extract was assayed according to Meir et al [108] with few modifications. Briefly, 50 µl of methanolic extract was mixed with 950 µl of 0.2 M phosphate buffer (pH 6.6) and 1% potassium ferricyanide (0.5 mL) and incubated for 20 min at 50 °C. TCA (0.5 mL, 10%) was added to the mixture, which was centrifuged at 12000 rpm for 10 min. To 0.5 mL of supernatant an equal volume of distilled water and 0.1 mL of 0.1% $FeCl_3$ solution was added. The reaction mixture was left for 10 min at room temperature, and the absorbance measured at 700 nm. Reducing activity was expressed as µg ascorbic acid equivalent antioxidant capacity (AEAC) g^{-1} of cabbage.

2.13.5 Hydroxyl radical scavenging assay

The deoxyribose method for determining the scavenging effect of the methanolic extract on hydroxyl radicals was performed according to earlier reported procedure [109]. The reaction mixture contained deoxyribose (28Mm), $FeCl_3$ (20Mm), EDTA (1Mm), H_2O_2 (20mM), ascorbate (1Mm) and different concentration of methanolic extract. The reaction mixture was incubated for 1hr at 37°C. Next 1% TCA and 2.8% TBA was added and incubated at 90°C for 30min. OD was measured at 532nm. Hydroxyl radical scavenging activity was expressed as µg GAE g^{-1} of cabbage.

2.13.6 Nitric oxide scavenging assay

The nitric oxide scavenging effects of methanolic extract was measured according to the method of Marcocci et al [110]. Briefly, 100µL of methanolic extract solution was added to 200µL of 10mM SNP solution and incubated at 25 °C for 150 min. To 100 µL of the above mixture 1 mL of Griess reagent was added. The absorbance immediately read at 546 nm. Nitric oxide radical scavenging activity was expressed as µg quercetin equivalent (QE) g^{-1} of cabbage.

2.13.7 Ascorbic acid content

Ascorbic acid content was quantified by both titrimetric and microfluorometric method of AOAC [111]

i) Titrimetric method

a) Assay: Total vitamin C content of cabbage was estimated in accordance with standard AOAC official titrimetric method [111]. Cabbage (10 gm) was extracted with 20% metaphosphoric acid in an omnimixture. The homogenate was centrifuged at 12000 rpm for 20 min. Reducing capacity of the supernatant was measured by titrating with 2,6 dichlorophenol indophenols (DCPIP). The end point of the reaction was detected by appearance of pink color by excess of the dye in the acidic solution. The same process was followed for standard ascorbic acid solutions of known

concentration (0.1–0.0015%). Ascorbic acid content was expressed as mg/100 g FW of cabbage of cabbage.

ii) Microfluorimetric method.

a) Assay: Total ascorbic acid content of cabbage was estimated in accordance with standard official microfluorometric method of AOAC[111]. 10 g of cabbage sample was extracted with 20 mL of 20% metaphosphoric acid and centrifuged at 12000 rpm for 20 min at 4 °C. A part of the supernatant was treated with activated charcoal (20 g/L) with vigorous shaking to convert ascorbic acid into DHA while the other part was kept aside to estimate the native DHA. The mixture was again centrifuged at 12000 for 10 min at 4 ◦C for removal of charcoal. Aliquots of the supernatant (500µL) were added to equal volumes of boric acid–sodium acetate solution (3% boric acid in 3.67 mol L^{-1} sodium acetate solution). The solution was left undisturbed for 15 min and the total volume was finally adjusted to 10 mL using milli Q water. This was the blank solution. To prepare the sample solution, another aliquot of 500 µL as well as charcoal untreated sample was mixed with an equal volume of sodium acetate solution (3.67 mol L^{-1}) and the final volume adjusted to 10mL using milli Qwater. A 0.4 mL aliquot of all the samples (charcoal treated, untreated and blank) was separately treated with 1 mL of o-phenylenediamine solution (0.02%), vortexed and incubated for 35 min at room temperature (RT). Ascorbic acid reacts with O-phenylenediamine to form a fluorescent conjugate. Total conjugate formed was measured (Excitation 350 nm; Emission 430 nm, Bandwidth 5 nm) using CS-5000 fluorimeter (Shimadzu Corporation, Japan). Same protocol was followed for standard ascorbic acid solutions of known concentration (0.1–0.0015%) to draw a standard curve. Linear regression was then used to determine the concentration of ascorbic acid in each sample.

2.14 Molecular studies

2.14.1 Isolation of total RNA

Procedure: RNA extraction was done using TRI reagent (Sigma, T 9424) as per manufacturer's instructions with some modifications. 100 mg of cabbage leaves were crushed with liquid nitrogen using a flame sterilized mortar and pastel. 1 mL of TRI reagent was added to it and the sample was crushed again. The mixture was kept undisturbed till it was fully thawed to form a pink liquid. The liquid was transferred to an eppendorf tube to which 200 µl of chloroform was added and vortexed vigorously for 15 sec. It was allowed to stand at RT for 5 min and then centrifuged at 14000 rpm for 15 min at 4 °C. The upper aquous layer was transferred carefully to a fresh tube to which 0.5 ml isopropanol was added. It was left undisturbed for 10 min at RT, and then further centrifuged at 12000 rpm for 15 min at 4 °C. The supernatant was removed carefully and RNA pellet washed with 1 ml of 75% ethanol by centrifuging at 12000 rpm for 15 min. The RNA pellet was dried briefly for 5-10 min under air. It was finally dissolved in 50 µl of diethylpyrocarbamate treated autoclaved water.

The quantity of RNA was measured using a NanoDrop 3300 spectrophotometer (Thermo Scientific, Waltham, MA). Absorbance was measured at 230, 260 and 280 nm. Samples showing a 260/280 and 260/230 ratio near to 2 were chosen for further studies. The integrity was checked by electrophoresis of total RNA (1 µg) on a 1.2% denaturing agarose gel.

2.14.2 cDNA preparation

Procedure: Three µg of the total RNA was reverse transcribed with Affinity script supermix for RT-PCR (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The reaction mixture contained:

RNA 3 µg

OligoDT 1.5 µl

Master mix 10 µl

The final volume was adjusted to 20 µl with water. The PCR cycling conditions consisted of 40 cycles with each cycle having following temperature program; 25 ºC for 5 min, 45 ºC for 5 min, 55 ºC for 5 min and 95 ºC for 5 min. cDNAs were stored at –20 ºC until till further use.

2.14.3 Primer optimization for quantitative real time-PCR

a) Primer designing: Primers were designed for different glucosinolate biosynthetic genes namely, *MYB 28, CYP79F1, CYP83A1, CYP83B1, TGG1, SOT 18, UGT74B1, SUR1* and *ACTIN2.*

Gene sequence and cDNA sequence were obtained from NCBI database. Nucleotide blast of the two sequences was done to get exonic and intronic region. Sequence corresponding to appropriate exonic region of the gene sequence were chosen from cds and feed into PRIMER 3 software to design the corresponding primers. The primers having similar Tm for forward and reverse primer were chosen. Primer blast was done for the designed primer with *Brassica oleracea* genome to confirm the specificity of the primer to the corresponding gene. The primer sequences thus obtained were finally given to Era scientific, Mumbai to design the required primers (Table 10)

b) Resuspending primers: Primers acquired were in the form of lyophilized film at the bottom of cryo tubes. They were dissolved in specific amount of autoclaved water as per manufacturer's instructions to make 100 µM stocks. These were further diluted to make 10 µM working solution.

c) Melting point (Tm) optimization: 5 µl of cDNA from control as well as treated samples were mixed to get a mixed cDNA sample. To optimize Tm of the primers PCR was run for each primer at different Tm. The reaction mix contained:

Mix cDNA	2 µl
Forward primer	1.5 µl
Reverse primer	1.5 µl
Water	5 µl
Master mix	10 µl

The PCR cycling conditions comprised 40 cycles, each cycle had following temperature program: 95 ºC for 30 s, 55 ºC for 30 s, and 72 ºC for 30 s. The PCR products were run on a 1.2% denaturing agarose gel and bands checked. A single band corresponding to the PCR product was observed for all the genes except *MYB28.* For *MYB28* PCR was run with Tm 46 ºC. The reaction mixture was same, the PCR cycling conditions comprised 40 cycles each comprising following temperature program; 95 ºC for 30 s, 46 ºC for 30 s, and 72 ºC for 30 s.

2.14.4 Quantitative Real Time-PCR

Procedure: The primer sets of glucosinolate biosynthetic genes as designed above were used for qRT-PCR. Actin gene (*ACTIN2*) was used as reference gene for normalization and quantification. It was verified that the level of actin was unchanged under all the given treatments. The PCR efficiency of the reference and target genes was also checked and found to be approximately equal in a range of 1.96–1.99. Real-time quantitative RT-PCR was carried out using a Corbett rotor gene 3000. Detection of real-time RT-PCR products was done using a SyBr Green 23 Master Mix kit (S 4320, Sigma), as per the manufacturer's instructions. The quantity of cDNA used as a template for PCR was 2.5 µg. The PCR cycling conditions comprised 40 cycles each

cycle had following temperature program; 95 ºC for 30 s, 55 ºC for 45 s, and 72 ºC for 30 s. For *MYB28*, cycling conditions were 40 cycles with each comprising 95 ºC for 30 s, 46 ºC for 45 s, and 72 ºC for 30 s. For each sample, reactions were set up in triplicate to ensure the reproducibility of the results.

At the end of each PCR run, a melting curve was generated and analysed with the dissociation curve software built into the Corbett rotor gene 3000. A relative expression ratio plot was generated using the software REST-MCS.

Table 10 List of primers used for qRT-PCR

Target gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
PAL	GCCGGAGTATCG	AGCCGCTCTGATC
ACTIN2	TCCAGGAATCGTTCACAG	GCTACAAAACAATGGGAC
MYB28	CCAAGGCGTGTTTATTAC	CCAATTCGCGAGGTTA
*CYP79F*1	AAGAAGGTGGTAAGGCTGCTGTT	AATGTGGCTACCTTTGGGAATGA
*CYP83A*1	GATTCCTCTCCTTATCCCTC	TAAACTCGTAGTCCGTGCCT
SOT18	ACGACGAGACCAAGACAGAATCAA C	GAGAACATCAACTTCAGGGAAGAAA
*UGT74B*1	CACCACTACCTACACCGCCTCCTCA	GCTCAAAGACGGTAAGCCACGGATA
SUR1	ACAATCCCTGTGGAAATGTCTACTC	ACAACCCATCCCTTAGATATGCC
TGG1	AGACCTCAAAGCATCTGGCA	CTCCTTATCTATGGAGCAAG

Chapter 3

RESULTS AND DISCUSSION

3.1 Screening of vegetables

Brassica vegetables are one of the most popular vegetables consumed worldwide. The inherent bioactive compounds like phenolics and glucosinolate make them a rich nutritional source. Epidemiological studies have shown that an adequate consumption of these vegetables has been associated with a reduced risk of various chronic diseases[1]. Brassica vegetables are characterized by their typical sulfurous flavour imparted by different groups of sulfur aroma compounds such as isothiocyanates, thiols and sulphides[76, 97]. Changes in these constituents can have significant impact on aroma quality of these vegetables. A preliminary screening of some Indian brassica vegetables was therefore carried out to assess changes in the above aroma compounds during radiation processing. Four commonly consumed brassica vegetables vegetables namely; cauliflower, radish, broccoli and cabbage were therefore selected. The aroma quality of each of these radiation processed vegetables was also further analyzed by a sensory panel using QDA.

Table 11 lists the major odor active sulfur compounds extracted by SPME and identified by GC/MS in the above vegetables. Allyl isothiocyanate (AITC) was the major compound identified in cabbage while dimethyl trisulfide was found to be the key volatile sulfur compound in cauliflower and broccoli. 3-(methylthio) propyl isothiocyanates was identified as the major volatile sulfur compound in radish. Gamma irradiation (2kGy) did not bring about significant change in the content of sulfur aroma compounds in any of these vegetables except cabbage. In cabbage a dose dependent increase was noted in the content of AITC. This compound due to its lower threshold and higher concentration is known to be the aroma impact compound in cabbage. An enhancement in quality as a consequence of increased AITC is thus expected. This was also further confirmed by sensory analysis of the above four vegetables.

Table 11 Effect of gamma irradiation on odor active sulphur compounds of different brassica vegetables.

Variety	Volatile (mg/kg) / Dose	Dimethyl disulfide	AITC	Dimethyl trisulfide	Dimethyl tetrasulfide	3-(Methylthio) propyl isothiocyanate
Cabbage	0kGy	1.1±0.2[a]	14.2±1.2[a]	1.5±0.4[a]	0.4±0.1[a]	1±0.1[a]
	0.5kGy	1.1±0.4[a]	25±2.1[b]	1.5±0.3[a]	0.4±0.1[a]	1±0.1[a]
	1kGy	1.1± 0.3[a]	36± 2.9[c]	1.5±0.2[a]	0.4±0.1[a]	1±0.1[a]
	2kGy	1.1± 0.2[a]	48±3.3[d]	1.5±0.4[a]	0.4±0.1[a]	1±0.8[a]
Cauliflower	0kGy	1.9± 0.2[a]	-	4.6±0.4[a]	1.6±0. 5[a]	-
	0.5kGy	1.9± 0.3[a]	-	4.8±0.7[a]	1.6±0.2[a]	-
	1kGy	1.8± 0.2[a]	-	4.7±0.5[a]	1.5±0.4[a]	-
	2kGy	1.9± 0.1[a]	-	4.6±0.8[a]	1.7±0.8[a]	-
Broccoli	0kGy	0.2± 0.1[a]	-	2.5±04[a]	1.2±0.[1a]	-
	0.5kGy	0.2±0.1[a]	-	2.6±0.7[a]	1.3±0.1[a]	-
	1kGy	0.2±0.1[a]	-	2.5±0.8[a]	1.2±0.1[a]	-
	2kGy	0.2±0.1[a]	-	2.4±0.6[a]	1.2±0.1[a]	-
Radish	0kGy	2.2± 0.3[a]	-	0.8±0.1[a]	-	4.5±1.2[a]
	0.5kGy	2± 0.4[a]	-	0.8±0.1[a]	-	4.5±1.1[a]
	1kGy	2.1± 0.1[a]	-	0.8±0.1[a]	-	4.6±1.2[a]
	2kGy	2.2± 0.6[a]	-	0.8±0.1[a]	-	4.4±1.3[a]

Data are expressed as mean ± standard deviation (n=9). Mean values in the same column for a vegetable bearing different superscripts are significantly different ($p < 0.05$).

Aroma quality of each of these radiation processed (2 kGy) vegetables was assessed by a sensory panel using QDA (Fig 14). The parameters analyzed were cabbage/cauliflower/broccoli/radish like odor and off odor. An enhancement in the characteristic cabbage like odor was detected in irradiated cabbage samples. No change in aroma quality was perceived in broccoli and cauliflower by the panel. A slight off odor was detected in radish due to irradiation. ***Since, radiation processing resulted in overall enhancement in aroma quality of cabbage, this vegetable was taken up for detailed investigation.***

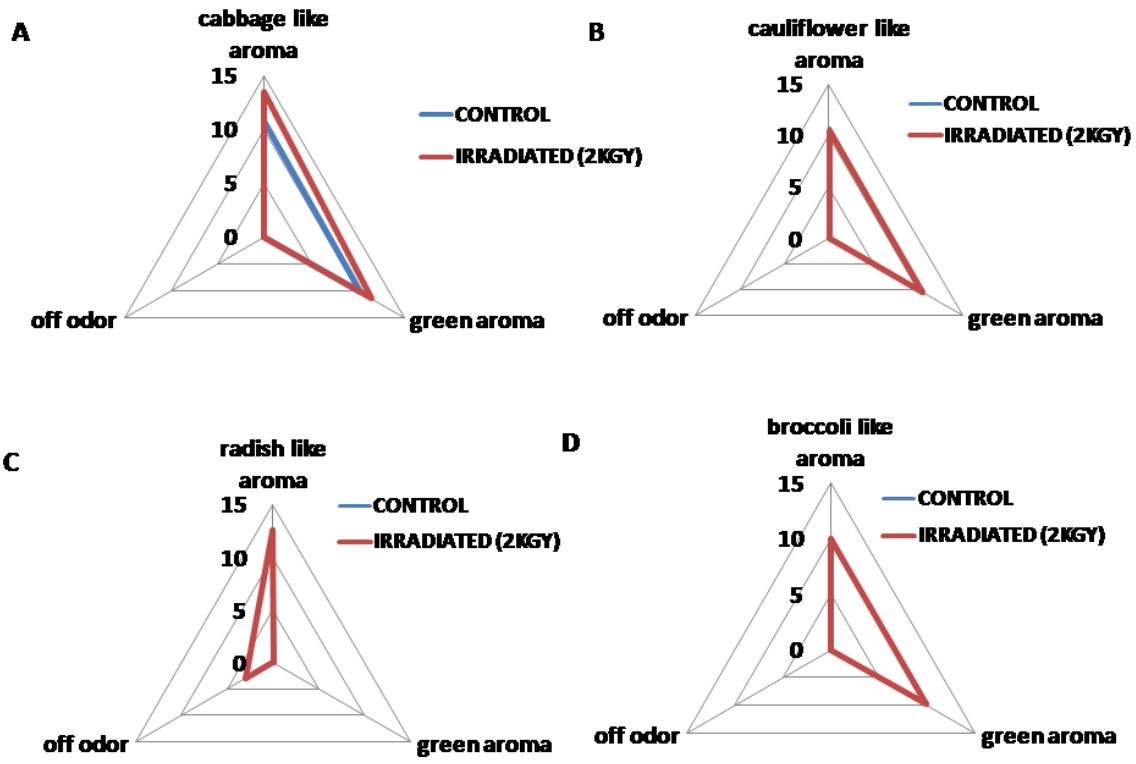


Fig 14 Quantitative descriptive analysis of control and radiation-processed sample immediately after irradiation. A)Cabbage B) Cauliflower C) Radish D) Broccoli

3.1.1 Volatile oil composition of cabbage

Cabbage leaves are characterized by a peculiar pungent odor and taste that is related mainly to the content of the glucosinolate degradation products, particularly the volatile isothiocyanates [76]. These compounds are one of the most characteristic metabolites produced by *Brassica* plants. Vegetables are also characterized by their green odors that impart fresh like character to these products [77]. Volatile aliphatic C_6 compounds including C_6 aldehydes and alcohols and their corresponding hexyl esters, each with their own delicately different odors, decisively contribute to these characteristic odors [77]. The intensity of these odor notes provide the state of freshness and thus is an important quality attribute of the vegetable.

Volatile aroma compounds were isolated by both SPME and Likens-Nickerson SDE apparatus. Two varieties of cabbage (BC-79 and NS-22) as well as market samples were used in the present study. Table 12 lists the major volatile compounds identified by these two techniques respectively. A higher content of volatile compounds was found in the isolate obtained by SDE. Similar results were observed by Chen et al in radish wherein a comparative analysis of aroma compounds obtained by different extraction methods indicated a better extraction of isothiocyanates by SDE than SPME [112]. Similarly in *Houttuynia cordata* the content of aldehydes like hexanal and decanal extracted by SDE was almost double than by SPME [113]. Higher extractability of SDE could account for this observation. Hence, SDE was used for further studies. Prolonged SDE can lead to generation of thermal artifacts like furfural. Hence, extraction was carried out for 1.5 hrs where maximum extraction of volatiles was observed with generation of no thermal artifacts. Table 13 lists the major volatile compound obtained from SDE in three varieties of cabbage. The nature of the compounds identified in different varieties is similar to that reported in

literature [76]. Qualitative and quantitative differences in the volatile constituents were noted between the varieties currently investigated. The content and pattern of volatiles are reported to vary according to plant species, cultivars and vegetable part, as well as with the developmental stage of the plant [76]. Among the various compounds identified, sulfur compounds and green leaf volatiles were the major constituents of the oil. Allyl isothiocyanate (AITC) was the major sulfur compound identified in all the varieties. This compound showed a wide variation in its distribution among the different varieties studied (Table 13). The market sample had the highest content of AITC, followed by BC-79 and NS-22. Variation in this major isothiocyanate among the different varieties can have a significant impact on their flavor quality. Other isothiocyantes identified such as 3-butenyl isothiocyanate and 3-(methylthio) propyl isothiocyanates, also showed a wide variation in their distribution among the different varieties. Their content was highest in market samples and lowest in NS-22. However, the impact of changes in these minor isothiocyanates on the overall odor quality of the vegetable can be assumed to be insignificant due to their far lower concentrations in the vegetable and higher odor threshold compared to AITC. Volatile sulfides such as dimethyl disulfide (DMDS) with a sulfurous cabbage like odor, dimethyl trisulfide (DMTS) with a sulfurous cauliflower like odor and dimethyl tetrasulfide (DMTES) having a garlic meaty odor are known to be odor active compounds of cooked Brassica species [88]. These compounds are derived either from (+)-S-methyl-L-cystein sulfoxide found in Brassica vegetables or formed by degradation of volatiles derived from glucosinolate break down. The content of these volatile sulfides also varied significantly among the three varieties (Table 13). A wide variation in the content of volatile sulfur compounds among cultivars and with the maturity has been reported in Brassica species.

Changes in the distribution of volatile sulfur compounds identified can have a significant impact on the aroma quality of the vegetable.

Table12 Aroma profile of volatiles as obtained from SDE and SPME extraction

Volatile	Content mg/kg (SPME)				Content mg/kg (SDE)			
	0 kGy	**0.5 kGy**	**1 kGy**	**2 kGy**	**0 kGy**	**0.5 kGy**	**1 kGy**	**2 kGy**
Dimethyl disulfide	1.08± 0.2 [a]	1.07± 0.4 [a]	1.06± 0.3 [a]	1.08± 0.2 [a]	1.9± 0.2 [a]	1.9± 0.3 [a]	1.8± 0.2 [a]	1.9± 0.1 [a]
n-Hexanal	1.37± 0.09 [a]	1.36± 0.08 [a]	1.37± 0.07 [a]	1.36± 0.08 [a]	2.09± 0.1 [a]	1.5± 0.08 [a]	1.4± 0.09 [a]	1.98± 0.08 [a]
trans-Hex-2-enal	0.61± 0.04 [a]	0.78± 0.03 [b]	0.99± 0.05 [c]	1.02± 0.09 [d]	1.18± 0.23 [a]	1.35± 0.4 [b]	1.81± 0.6 [c]	2.01± 0.15 [d]
cis-Hex-3-enol	0.51± 0.06 [a]	0.5± 0.05 [a]	0.51± 0.06 [a]	0.5± 0.05 [a]	2.77± 0.53 [a]	1.8± 0.3 [a]	1.6± 0.4 [a]	2.48± 0.65 [a]
AITC	14.2± 1.2 [a]	25.13±2.1 [b]	36.78± 2.9 [c]	48.13± 3.3 [d]	31.5± 3.11 [a]	35± 1.3 [b]	48.2± 3.1 [c]	52.92± 6.5 [d]
Dimethyl trisulfide	1.54± 0.4 [a]	1.52± 0.3 [a]	1.53±0.2 [a]	1.51± 0.4 [a]	4.22± 0.99 [a]	4.8± 0.7 [a]	4.7± 0.5 [a]	3.92± 1.01 [a]
But-3-enyl isothiocyanate	1.9± 0.5 [a]	1.7± 0.8 [a]	1.7± 0.9 [a]	1.8± 0.7 [a]	3.01± 0.73 [a]	2.1± 0.11 [a]	2.5± 0.12 [a]	2.9± 0.8 [a]
Dimethyl tetrasulfide	0.41± 0.03 [a]	0.39± 0.05 [a]	0.43± 0.02 [a]	0.42± 0.04 [a]	0.43± 0.05 [a]	0.6± 0.2 [a]	0.5± 0.4 [a]	0.39± 0.08 [a]
3-(Methylthio) propyl isothiocyanate	0.99± 0.06 [a]	0.97± 0.08 [a]	0.96± 0.07 [a]	0.98± 0.8 [a]	1.02± 0.09 [a]	1.11± 0.7 [a]	1.12± 0.9 [a]	0.91± 0.19 [a]

Data are expressed as mean ± standard deviation (n=9). Mean values in the same row for a SPME or SDE bearing different superscripts are significantly different ($p < 0.05$).

SDE content: Content of each volatile in the oil obtained by Steam distillation extraction; mg of each compound per Kg of cabbage taken.

SPME content: Content of each volatile as obtained by solid phase microextraction; mg of each compound per Kg of cabbage taken.

Among the GLVs significant amounts of n-hexanal, *trans*-hex-2-enal and *cis*-hex-3-enol was noted. These compounds with a characteristic green odor are associated with sensory perception of freshness [77]. n-Hexanal, characterized by green, grassy odor note, is reported to be the key odor compound of fresh broccoli florets while *trans*-hex-2-enal and *cis*-hex-3-enol, possessing fresh green and leafy aroma are reported to play a relatively important role in cabbage flavour [97]. The above C6 aldehydes and alcohols that form part of the GLVs is known to be formed via the lipoxygense pathway from unsaturated fatty acid precursors namely linoleic and linolenic acids liberated mainly from galactolipids. The content of n-hexanal and *trans*-hex-2-enal was highest in the market samples. The concentration of these two compounds was, however, higher in NS-22 than BC-79. No significant difference in the distribution of *cis*-hex-3-enol was noted between the different varieties tested. Earlier work has demonstrated that *trans*-hex-2-enal and *cis*-hex-3-enol were formed almost exclusively from the outer and older leaves with virtually none from the inner leaves of cabbage [114]. C6 aldehydes formation in leaves has also been reported to be under developmental control and therefore dependent on leaf age [114]. The changes in the distribution of GLVs observed could thus be possibly explained by the variation in the maturity between the various varieties studied.

3.1.1.1 Effect of radiation processing on volatile constituents of cabbage

Processing by high energy ionizing radiation is an important post-harvest preservation techniques currently practiced worldwide. The process has recently been recommended for microbial decontamination of fresh leafy green vegetables of the Brassica species such as lettuce [55]. FDA, USA allows the use of ionizing radiation up to 4 kGy to make these products safer and delay spoilage [55]. No published literature, however, exists on the effects of such a processing method on the content of GLVs and other aroma imparting compounds

in vegetables of the Brassica species. In order to determine the optimum dose that could be allowed for treatment of these vegetables the effect of radiation processing at various doses on the sensory acceptability was initially investigated by a trained panel. The sensory panel could clearly detect off odors at doses beyond 2.5 kGy in cabbage. Also softening in texture at 3 kGy was noted. Hence the samples exposed to doses beyond 2 kGy were not investigated. Radiation processing of vegetables usually involves exposure to doses in the range of 0.5 to 2 kGy for microbial decontamination [51]. Hence, the radiation doses applied in the present studies ranged from 0.5 to 2kGy. Table 14 shows the effect of radiation processing at various doses on the composition of the volatile oil of NS-22 variety of cabbage. Except for AITC and *trans*-hex-2-enal, the content of other constituents identified were unaffected by radiation processing. An increase in the content of AITC was noted immediately after irradiation. The extent of increase was found to be radiation dose dependent with highest increase at 2kGy. To further confirm these observations, effect of radiation processing was also studied on market sample and BC-79 variety of cabbage exposed to a dose of 2kGy. Table 13 compares the effect of gamma irradiation on three varieties of cabbage at 2kGy. A radiation induced enhancement in AITC was noted in all the three varieties studied depending on the variety. The highest increase was noted for NS-22 with an increase in content by 80% followed by the market sample (68%) and variety BC-79 (44.5%). Sinigrin, known to be the precursor of AITC, is a major glucosinolate of cabbage [41]. Changes in the distribution of this compound among the different varieties and its breakdown during radiation processing could possibly account for the variation in the content of AITC observed.

Post harvest storage is known to influence volatile content of vegetables. A decrease in isothiocyanate content during storage has been previously reported by Engel et al [78]. In the present work the increase in AITC content was not found to be significantly affected by post-

irradiation storage (10 °C) with a slight decrease in the content during storage (Fig 15). The content of this compound was, however, considerably higher than the control samples throughout the storage period studied. To the best of our knowledge this is the first report on the gamma radiation induced enhancement in AITC content in cabbage. As AITC is known to contribute to the characteristic odor and taste of the vegetable, its enhanced formation during radiation processing can have a significant impact on its flavor quality. AITC is also reported to be protective against bladder and colon carcinoma. Hence, an improvement in its content can also significantly improve the nutraceutical value of the product.

As GLVs are the other major constituents of volatile oil, changes in these components in the radiation processed vegetable was further investigated. The effect of radiation processing (0.5-2kGy) on the content of the three GLVs identified is shown in Table 14. Among the three GLVs, the content of *trans*-hex-2-enal increased significantly immediately after irradiation while that of n-hexanal and *cis*-hex-3-enol was unaffected. Similar to AITC, an increase in content of *trans*-hex-2-enal with dose was noted. Table 13 compares the effect of radiation processing on three varieties of cabbage. The increase in *trans*-hex-2-enal was found to be variety dependent with the highest increase noted in NS-22 (78%) followed by the market sample (70.3%) and BC-79 (40.6%). *trans*-hex-2-enal is known to be an oxidative product of linolenic acid formed via the lipoxygenase pathway [77]. Variation in the content of linolenic acid liberated as a result of lipid radiolysis in the different varieties could possibly explain the observed variation in *trans*-hex-2-enal content. UV irradiation of tomato fruits and leaves was shown to increase the production of n-hexanal as a result of enhanced LOX and HPL activity [77]. Byun, et al also reported an increased *trans*-hex-2-enal content in soybeans due to gamma irradiation at a dose above 10 kGy with as high as 5 times increase at 100 kGy [122]. Fan and Sokorai on the other hand observed an increase in

trans-hex-2-enal content of cilantro during post-harvest storage with no significant effect on the content of this compound on irradiation [146]. The content of *trans*-hex-2-enal formed was also found to vary with post-irradiation storage. A rapid decrease in *trans*-hex-2-enal content of the irradiated vegetable from its initial value was noted on storage up to a period of one day beyond which its concentration was comparable to that of the non-irradiated sample (Fig 15). An immediate increase followed by subsequent lowering in GLV content in *Arabidopsis* on wounding has been reported by Matsui et al [84]. Similar results have been observed by several other researchers on pathogen and herbivore attack on green leaves emphasizing the role of GLVs as typical wound signals. A similar phenomenon may be acting in radiation processed cabbage, however, this observation needs further investigation. As irradiation was not found to affect the content of other volatiles identified in the present study, the mechanism of increased AITC and *trans*-hex-2-enal was further investigated.

Table 13 Effect of irradiation (2 kGy) on volatile oil composition for three different varieties of cabbage.

Volatiles	NS-22 (mg/kg)		BC-79 (mg/kg)		Market sample (mg/kg)	
Common name	control	irradiated	Control	irradiated	control	irradiated
Dimethyl disulfide	0.21 ± 0.03 [a]	0.28 ± 0.09 [a]	1.71 ± 0.09 [a]	1.78 ± 0.19 [a]	1.47 ± 0.09 [a]	1.27 ± 0.61 [a]
2-Penten-1-ol	0.049 ± 0.007 [a]	0.047 ± 0.01 [a]	0.07 ± 0.03 [a]	0.07± 0.06 [a]	-	-
Hexanal	1.66 ± 0.09 [a]	1.62 ± 0.09 [a]	0.39 ± 0.03 [a]	0.39 ± 0.07 [a]	2.09 ± 0.1 [a]	1. 98 ± 0.8 [a]
trans-Hex-2-enal	1.1 ± 0.11 [a]	1.96 ± 0.16 [b]	0.64 ± 0.02 [a]	0.90 ± 0.02 [b]	1.18 ± 0.23 [a]	2.01 ± 0.15 [b]
cis-Hex-3-enol	2.6 ± 0.54 [a]	2.1 ± 0.89 [a]	2.3 ± 0.32 [a]	2.9 ± 0.12 [a]	2.77 ± 0.53 [a]	2.48 ± 0.65 [a]
Allyl isothiocyanate	9.4 ± 1.83 [a]	16.89 ± 3 [b]	28.3 ± 1.9 [a]	40.9 ± 1.61 [b]	31.5 ± 3.11 [a]	52.92 ± 6.5 [b]
Dimethyl trisulfide	1.66 ± 0.09 [a]	1. 6 ± 0.19 [a]	14.5 ± 2.61 [a]	13.6 ± 1.76 [a]	4.22 ± 0.99 [a]	3.92 ± 1.01 [a]
But-3-enyl isothiocyanate	1.63 ± 0.33 [a]	1.89 ± 0.43 [a]	2.26 ± 1.09 [a]	2.06± 0.98 [a]	3.01 ± 0.73 [a]	2.9 ± 0.8 [a]
Dimethyl tetrasulfide	0.11 ± 0.005 [a]	0.11 ± 0.06 [a]	0.98 ± 0.19 [a]	0.93 ± 0.17 [a]	0.43 ± 0.05 [a]	0.39 ± 0.08 [a]
3-(methylthio)propyl isothiocyanates	0.007 ± 0.001 [a]	0.0074 ± .005 [a]	0.34 ± 0.09 [a]	0.38 ± 0.1 [a]	1.02 ± 0.09 [a]	0.91 ± 0.19 [a]

Data are expressed as mean ± standard deviation (n=9). Mean values in the same row for a variety bearing different superscripts are significantly different ($p < 0.05$).

Table 14 Effect of radiation doses on volatile oil composition of NS-22 variety of cabbage.

Volatiles (mg/Kg)	Control	0.5 kGy	1 kGy	2kGy
Dimethyl disulfide	0.21 ± 0.03 [a]	0.28 ± 0.09 [a]	0.22 ± 0.09 [a]	0.25 ± 0.09 [a]
2-Penten-1-ol	0.049 ± 0.007 [a]	0.044 ± 0.01 [a]	0.46 ± 0.03 [a]	0.047 ± 0.01 [a]
Hexanal	1.66 ± 0.09 [a]	1.62 ± 0.09 [a]	1.67 ± 0.03 [a]	1.62 ± 0.09 [a]
trans-Hex-2-enal	1.1 ± 0.11 [a]	1.96 ± 0.16 [b]	0.64 ± 0.02 [c]	1.96 ± 0.16 [d]
cis-Hex-3-enol	2.6 ± 0.54 [a]	2.1 ± 0.89 [a]	2.3 ± 0.32 [a]	2.1 ± 0.91 [a]
Allyl isothiocyanate	9.4 ± 1.83 [a]	11.89 ± 3 [b]	14.3 ± 1.9 [a]	16.89 ± 3 [b]
Dimethyl trisulfide	1.66 ± 0.09 [a]	1. 6 ± 0.19 [a]	1.65 ± 2.61 [a]	1. 62 ± 0.19 [a]
But-3-enyl isothiocyanate	1.63 ± 0.33 [a]	1.69 ± 0.43 [a]	1.66 ± 1.09 [a]	1.69 ± 0.43 [a]
Dimethyl tetrasulfide	0.11 ± 0.005 [a]	0.121 ± 0.06 [a]	0.18 ± 0.19 [a]	0.11 ± 0.06 [a]
3-(methylthio) propyl isothiocyanates	0.007 ± 0.007 [a]	0.0074 ± .005[a]	0.007 ± 0.09 [a]	0.0074 ± .005[a]

Data are expressed as mean ± standard deviation (n=9). Mean values in the same row bearing different superscripts are significantly different ($p < 0.05$).

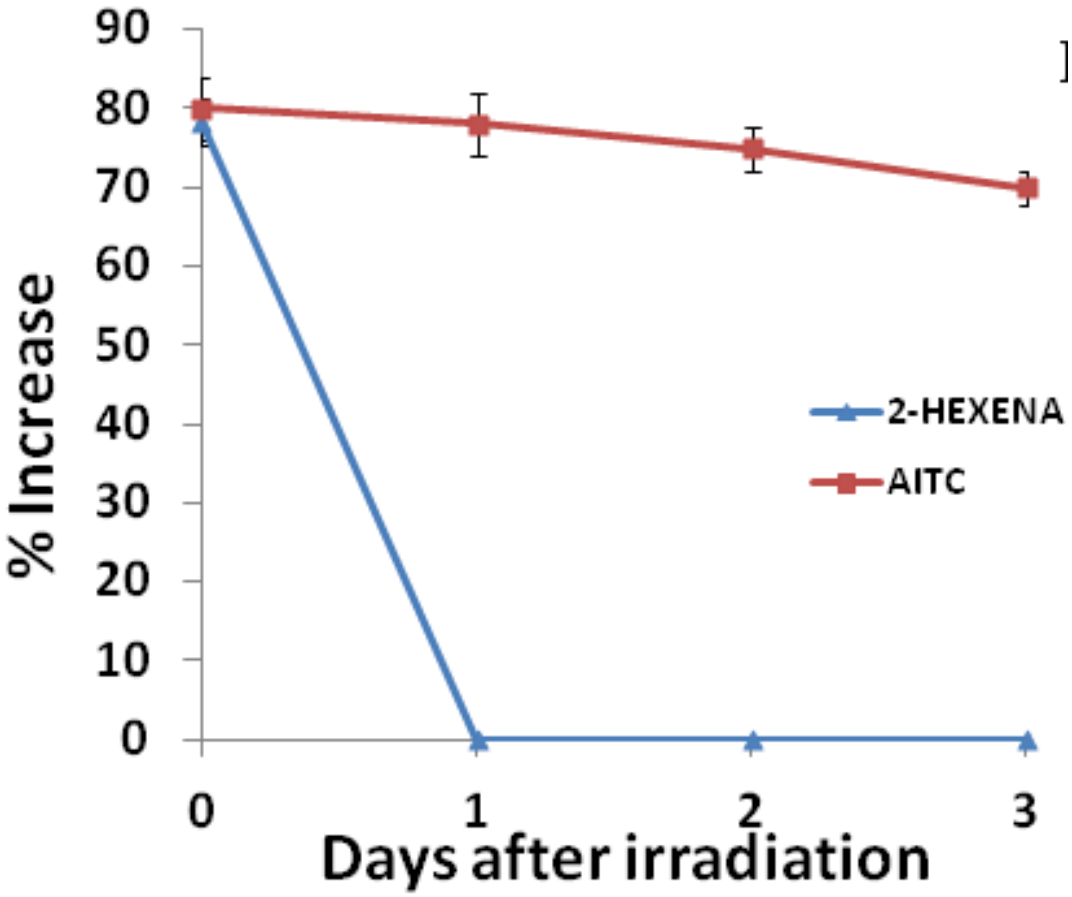


Fig. 15 Effect of storage after irradiation (2kGy) on AITC and 2-hexenal content. (zero on Y axis represents control value).

3.2 Mechanism of enhancement of GLVs

GLVs are responsible for imparting sensory perception of freshness to vegetables. Very few reports exist on the effect of biotic and abiotic stress on GLV content in vegetables. Wound induced enhancement in GLV content has been reported by Matsui et al [115] in Arabidopsis. An increase in GLVs was also seen with increasing temperature due to cell wall rupture [92]. Peroxidation of cell wall lipids by ozone has been reported to lead to emission of GLVs [92]. Similar results were also seen for UV treatment and drought stress [92]. However no reports have dealt with gamma irradiation induced change in GLV content.

The GLVs i.e. C6 aldehydes and alcohols are the products of Lipoxygenase pathway of lipid metabolism [77]. Membrane lipids when acted upon by the enzyme lipase release free fatty acids. The unsaturated fatty acids particularly the linoleic acid and linolenic acid are converted to their hydroperoxides by the enzyme lipoxygenase which are further cleaved by hydroperoxide lyase to release the GLVs. In the present study an enhancement in *trans*-hex-2-enal was noted in the irradiated samples. This compound is known to be formed from linolenic acid by the enzymes of lipoxygenase pathway. Hence, studies on mechanism of GLV enhancement by gamma irradiation were carried out to determine the effect of irradiation on lipid composition as well as on the enzymes of lipoxygenase pathway.

3.2.1 Lipid Composition of cabbage

3.2.1.1 Lipid species

Composition of neutral and polar lipids composition was studied in three cabbage varieties, namely, NS-22, BC-79 and market sample. Figure 16 A depicts the TLC chromatograms of neutral lipids in the cabbage sample. Fatty acid esters, triacylglycerol (TAG), free fatty acids (FFA) and sterols were identified as the major neutral lipid constituents in cabbage. Table 15 provides the quantitative distribution of the amount of each lipid species present in the

different varieties. TAG was identified as one of the major neutral lipid present. TLC chromatogram of galactolipids is depicted in Fig 16B. Monogalactosyldiacyl glycerol (MGDG) and digalactosyldiacylglycerol (DGDG) were found to be the predominant galactolipid species identified in cabbage. The major phospholipids identified include phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylcholine (PC) (Fig 16C). Triacylglycerols are reported to be the main constituents in Brassica oils. Peng et al in their earlier studies on the lipid composition of cabbage have reported the presence of neutral lipids, glycolipids and phospholipids as the major lipid constituents of the vegetable accounting for 51.02%, 40.78% and 8.18% of the oil. The distribution of various lipid species identified in the present study is comparable to the reported literature values. The lipid isolated from green vegetables mainly corresponds to those present in the thylakoid membranes of chloroplast. Lipid profile of the chloroplast membrane is known to be dominated by galactolipids and neutral lipids with phospholipids representing only a small fraction. This could account for a higher galactolipid and neutral lipid content in the present study. The content of phospholipids was comparable in all the three varieties. However, the content of other lipid constituents varied considerably depending on the variety (Table 15). BC-79 variety had the highest TAG, fatty acid ester and galactolipid (MGDG and DGDG) content. On the other hand, the content of FFA and sterols as well as phospholipids identified was highest in the market samples. Variations in these lipid species can have a significant impact on the content of precursor fatty acids liberated during radiation processing and thus on the content of GLVs in the volatile profile of the vegetable.

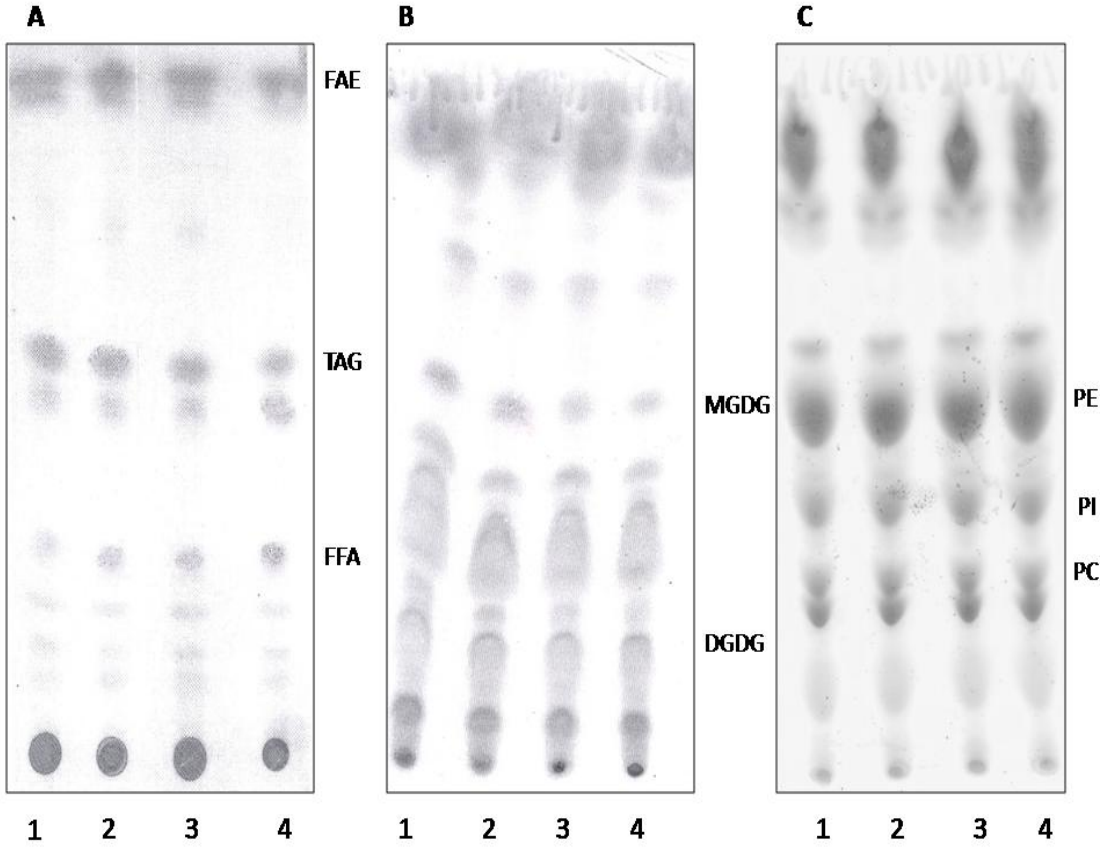


Fig 16 A. TLC of neutral lipids. B. TLC of galactolipids. C. TLC of phospholipids

1-Control, 2- 0.5 kGy, 3- 1kGy, 4- 2 kGy.

3.2.1.2 Fatty acid composition

Table 15 also lists the nature of fatty acids and their content in different cabbage varieties. Linolenic acid was the major fatty acid in all the varieties followed by linoleic and palmitic acid. The composition of fatty acids is similar to that reported earlier by Peng. The content of linolenic acid has been reported to increase with leaf age. The significant variation in this fatty acid among the different varieties observed here thus reflects their varying maturity. Amongst all the fatty acids released due to lysis of membrane lipids, linolenic acid is the precursor of *trans*-hex-2-enal. Hence the nature of the fatty acids in MGDG and TAG, the major lipid species of cabbage, and their contribution to the total fatty acid pool was of interest. Linolenic acid was found to be the major fatty acid in both MGDG and TAG (Table 16) with a higher content in MGDG. Thus a significant contribution of galactolipids to the linolenic acid content to the total fatty acid profile was inferred.

3.2.2 Effect of radiation processing on lipid constituents

3.2.2.1. Effect on lipid species

Effect of radiation processing (0.5-2 kGy) on the content of various lipid species identified in NS-22 variety of cabbage is shown in Table 17. MGDG was found to be the lipid species most sensitive to radiation processing with a considerable decrease in its content during irradiation. Radiation processing was, however, not found to significantly affect the content of DGDG. MGDG has been reported to be the lipid species most sensitive to stress [99]. DGDG: MGDG ratio forms an important parameter for maintaining membrane stability. Under stress, an increase in DGDG: MGDG ratio can maintain the membrane stability and fluidity necessary for biological function of chloroplastic membranes. In drought stressed plants, an increase in the DGDG: MGDG ratio was demonstrated, that served in maintaining bilayer

conformation and fluidity [117]. A similar stabilizing effect in response to radiation stress could possibly explain the lowering of MGDG levels in the leaves. Oxidative stress has also been reported to induce a drastic decrease in MGDG. The content of TAG also showed a considerable decrease albeit lower than MGDG in the irradiated vegetable. A decrease in TAG content as a consequence of radiolysis in irradiated nutmeg was demonstrated by us earlier [118]. Pai and Gaur in their report on the effect of gamma irradiation on the functioning of bean hypocotyls mitochondria have shown a high sensitivity of phospholipids particularly PC and PE to gamma irradiation [119]. In the present study, however, no significant effects of radiation on phospholipids were observed. This could possibly be due to the lower phospholipid content in cabbage compared to neutral and galactolipids. A linear decrease of TAG from 13.16 to 9.52 mg/kg and MGDG from 10.14 to 5.6 mg/kg was noted when the vegetable was irradiated in the dose range from 0.5 kGy -2 kGy. The enhanced free fatty acid content observed (Table 15) in irradiated (2 kGy) samples in all varieties and their increase with dose (Table 17) as demonstrated in NS-22 variety further supports the breakdown of the different lipid species such as MGDG and TAG during radiation processing. Table 15 compares the effect of radiation processing (2 kGy) on NS-22 variety of cabbage with BC-79 and market sample. Similar observations were made in all the three varieties. Gamma irradiation resulted in a decrease in MGDG and TAG content with a subsequent increase in free fatty acid content. The extent of this decrease however depended on the variety. The highest reduction of MGDG was in NS-22 (62%) followed by market variety (39%) and BC-79 (31%). The content of TAG decreased by 32.3% in irradiated NS-22 while the corresponding decrease in market variety and BC-79 was 27.8% and 12.6% respectively.

Table 15 Effect of irradiation (2 kGy) on lipid composition in 3 different varieties of cabbage**.**

Lipid species	NS 22		BC-79		Market sample	
(mg/kg)	Control	irradiated	Control	irradiated	Control	Irradiated
MGDG	14.74 ± .11[a]	5.6 ± 0.97[b]	17.12 ±0.91[a]	11.79 ± 0.41[b]	15.11 ±0 .31[a]	9.2 ± 0.12[b]
DGDG	3.41 ±0.11[a]	3.04 ±0.3[a]	12.98 ±0.8[a]	13.02 ±0.04[a]	10.12 ±0.22[a]	9.98 ±0.16[a]
TAG	13.98 ±0.13[a]	9.46 ± 0.39[b]	20.45 ± 1[a]	17.88 ± 0.76[b]	16.99 ±0.81[a]	12.26 ± 0.64[b]
FAE	1.58 ± 0.14[a]	1.97 ± 0.62[a]	3.18 ± 0.09[a]	3.01 ± 0.12[a]	1.98 ± 0.09[a]	2.08 ± 0.19[a]
FFA	5.5 ± 0.11[a]	7.86 ± 0.14[b]	5.8 ± 0.09[a]	6.38 ± 0.05[b]	7.5 ± 0.04[a]	9.3 ± 0.1[b]
Sterol	5.6 ± 0.07[a]	5.1 ± 0.19[a]	6.1 ± 0.44[a]	6.5 ± 0.3[a]	6.6 ± 0.2[a]	6.9 ± 0.15[a]
PE	1.01 ± 0.07[a]	0.98 ± 0.1[a]	2.31± 0.12[a]	1.99 ± 0.39[a]	2.6 ± 0.4[a]	2.1 ± 0.42[a]
PI	1.1 ± 0.2[a]	1.2 ± 0.3[a]	2.1 ± 0.21[a]	1.98 ± 0.9[a]	2.89 ± 0.3[a]	2.85 ± 0.09[a]
PC	0.98 ± 0.07[a]	1.01 ± 0.05[a]	1.56 ± 0.76[a]	1.69 ± 0.12[a]	2.1 ± 0.07[a]	1.98 ± 0.1[a]
Myristic Acid	0.19 ±0.005[a]	0.22 ±0.06[a]	0.13 ± 0.007[a]	0.14 ± 0.006[a]	0.29 ± 0.019[a]	0.3± 0.002[a]
Pentadecanoic Acid	0.10 ±0.011[a]	0.11 ±0.004[a]	0.12 ± 0.022[a]	0.13 ± 0.034[a]	0.17 ± 0.018[a]	0.18 ± 0.005[a]
Palmitic ic Acid	1.9 ± 0.034[a]	1.9 ± 0.036[a]	1.3 ± 0.04[a]	1.3 ± 0.06[a]	2.1 ± 0.023[a]	1.9 ± 0.033[a]
Stearic Acid	0.019 ±0.003[a]	0.019 ± 0.002[a]	0.016 ± 0.002[a]	0.016 ± 0.005[a]	0.018 ± 0.001[a]	0.016 ± 0.011[a]
Oleic Acid	0.082 ±0.004[a]	0.13 ± 0.007[b]	0.062 ± 0.006[a]	0.11 ± 0.002[b]	0.1 ± 0.007[a]	0.18 ± 0.005[b]
Linoleic Acid	0.98 ± 0.05[a]	0.99 ± 0.03[a]	1.05 ± 0.02[a]	1.11± 0.09[a]	1.56 ± 0.11[a]	1.51 ± 0.18[a]
Linolenic Acid	2.9 ± 0.07[a]	1.7 ± 0.04[b]	4.24 ± 0.09[a]	3.49 ± 0.11[b]	3.35 ± 0.07[a]	2.21 ± 0.06[b]

Data are expressed as mean ± standard deviation (n=9). Mean values in the same row for a variety bearing different superscripts are significantly different ($p < 0.05$).

Table 16 Fatty acid composition of TAG and MGDG

Fatty acid	From TAG hydrolysis (%)	From MGDG hydrolysis (%)
Myristic Acid	3.1±0.1	5.1±0.3
Pentadecanoic Acid	3.7±0.3	2.7±0.2
Palmiticic Acid	12.8±1.4	19.8±2.4
Stearic Acid	3.1±0.2	3.5±0.2
Oleic Acid	4.5±0.4	4.2±0.1
Linoleic Acid	12.8±2.1	8.8±1.1
Linolenic Acid	26±4.1	38±5.1

Table 17 Effect of gamma irradiation (0.5-2 kGy) on on lipid profile NS-22 variety of cabbage

Lipid species (mg/kg)	control	0.5 kGy	1 kGy	2 kGy
MGDG	14.74 ± 2.11[a]	10.6 ± 0.97[b]	8.12 ±0.91[c]	5.6 ± 0.97[d]
DGDG	3.41 ±0.11[a]	3.04 ±0.3[a]	3.18 ±0.8[a]	3.14 ±0.3[a]
TAG	13.98 ±0.13[a]	12.46 ± 0.39[b]	10.45 ± 0.1[c]	9.46 ± 0.39[d]
Fatty acid ester	1.58 ± 0.14[a]	1.57 ± 0.62[a]	1.18 ± 0.09[a]	1.97 ± 0.62[a]
FFA	5.5 ± 0.11[a]	6.09 ± 0.14[b]	6.8 ± 0.09[c]	7.86 ± 0.14[d]
Sterol	5.6 ± 0.07[a]	5.1 ± 0.19[a]	5.8 ± 0.44[a]	5.1 ± 0.19[a]
Phosphatidyl-Ethanolamine	1.01 ± 0.07[a]	1.18 ± 0.1[a]	1.31± 0.12[a]	0.98 ± 0.1[a]
Phosphatidyl-Inositol	1.1 ± 0.2[a]	1.2 ± 0.4[a]	1.1 ± 0.21[a]	1.2 ± 0.3[a]
Phosphatidyl-Choline	0.98 ± 0.07[a]	1.01 ± 0.05[a]	1.06 ± 0.76[a]	1.06 ± 0.05[a]
Myristic Acid	0.19 ±0.005[a]	0.22 ±0.06[a]	0.23 ± 0.007[a]	0.22 ±0.06[a]
Pentadecanoic Acid	0.10 ±0.011[a]	0.11 ±0.004[a]	0.12 ± 0.022[a]	0.11 ±0.008[a]
Palmiticic Acid	1.9 ± 0.034[a]	1.9 ± 0.036[a]	1.8 ± 0.04[a]	1.9 ± 0.056[a]
Stearic Acid	0.019 ± 0.003[a]	0.019 ± 0.002[a]	0.019 ± 0.004[a]	0.019 ± 0.002[a]
Oleic Acid	0.082 ± 0.004[a]	0.08 ± 0.007[a]	0.082 ± 0.006[a]	0.13 ± 0.007[b]
Linoleic Acid	0.98 ± 0.05[a]	0.96 ± 0.03[a]	0.95 ± 0.02[a]	0.99 ± 0.03[a]
Linolenic Acid	2.9 ± 0.07[a]	2.4 ± 0.04[b]	2.00 ± 0.09[c]	1.7 ± 0.04[d]
Eicosanoic Acid	0.02 ± 0.03[a]	0.021 ± 0.04[a]	0.026 ± 0.06[a]	0.021 ± 0.04[a]

Data are expressed as mean ± standard deviation (n=9). Mean values in the same row for a variety bearing different superscripts are significantly different ($p < 0.05$).

3.2.2.2 Effect on fatty acids

The effect of radiation processing on the content of fatty acid in the three varieties of cabbage is shown in Table 15. A significant decrease in linolenic acid content was observed in the radiation processed samples of all the three varieties studied (Table 15). A decrease in content of this acid by 41.3%, 34% and 17.6% was noted in NS-22, market sample and BC-79 respectively. Linoleic and linolenic acids are the major fatty acids demonstrated to be the precursors in the formation of volatile aliphatic C_6 aldehydes and alcohols, also termed as GLVs. Studies on *Arabidopsis* have shown an extensive decrease in amounts of galactolipids after homogenization with an corrwesponding increase in GLVs [91]. This study revealed that a lipase, specific to galactolipids, with high specificity towards MGDG, was involved in the formation of aldehydes upon disruption of Arabidopsis leaves [91]. Galactolipids as preferential substrate of lipase over phospholipids and triglycerides in GLV production has also been reported [91]. In the present study an enhanced break down of MGDG compared to other lipid species and its contribution to the total fatty acid profile suggests the possible role of this galactolipid in contributing to the enhanced *trans*-hex-2-enal content during radiation processing. Linolenic acid is reported to be the precursor of *trans*-2-hexanals and *cis*-hex-3-enols [77]. In the present study the decrease in linolenic acids was found to be linearly correlated (R^2 = 0.99) with the increased *trans*-2-hexenal content in the irradiated samples (Fig 17). Thus an enhanced formation of linolenic acid as a result of radiolysis and a consequent oxidation of the liberated fatty acid to *trans*-2-hexenal via lipoxygenase pathway could be inferred. It may be noted here that the content of other GLVs such as *cis*-hex-3-enol and hexanal were unaffected by radiation processing. *cis*-hex-3-enol is normally formed by reduction of 3-hexenal via alcohol dehydrogenase [77]. However, 3-hexenal was not detected in the volatile oils presently studied. This could explain the absence of any enhancement in 3-

hexenol as a result of radiation processing. Hexanal is reported to be formed from linoleic acid via the LOX pathway. No change in linoleic acid content was noted in the irradiated samples (Table 15) in the present study thus explaining the absence of enhanced hexanal as a result of radiation processing.

3.2.3 Effect of radiation processing on the enzymes in the LOX pathway

An increased formation of *trans*-2-hexenal in the vegetable immediately after gamma irradiation suggested the possible activation of the enzymes such as acyl hydrolase, lipoxygenase or HPLs [77]. It was therefore of interest to understand the role of these enzymes in enhancing the content of the aldehyde during processing. Lipase activity of extracts was studied in all the cabbage samples subjected to three different radiation doses (0.5, 1 and 2 kGy). Most of the protocols used for analysis of lipase activity are substrate specific. In the present study p-nitophenol blue was used as substrate for lipase activity as it can act as substrate for all the lipases present in the cell [100]. Activities of the enzymes ranged from 0.019 to 0.021 micromol/min/g fresh weight (FW) (Table 18). Lipases, especially galactolipases, are known to be induced by salt and mechanical stress [99]. However, no significant difference in lipase activity was observed between control and irradiated samples (Table 18). Zhuang, et al have earlier reported the role of 18:3 rich galactolipids as the possible direct substrate for LOX/HPL without the need for lipases for production C6 aldehyde [120]. Thus the limited role of this enzyme in the production of hexenal in the present study could be inferred. The effect of radiation treatment at the above doses on the activities of lipoxygenase and HPL were therefore further examined. Among the stress factors investigated, wounding, jasmonic acid treatment, or pathogen attack are reported to induce LOXs and HPLs [121]. Byun et al. have earlier reported a negative correlation between the irradiation dose and the lipoxygenase activity [122]. They reported a

71% inhibition of lipoxygenase activity when soybeans were irradiated at 100 kGy. In the present study, LOX activity ranged from 0.88 to 1.05 µmol/min/g FW (Table 18) while HPL activity was found to be between 1.32 to 1.45 µmol/min/g FW (Table 18) at the three doses investigated indicating no significant effect of radiation on the activity of these enzymes. Activities were also determined at different time intervals after irradiation (1hour, 5 hour and 1 day). No change in the enzyme activity was observed at all the intervals studied. Thus radiation processing was found to have no impact on the activity of the enzymes of the LOX pathway. Further, addition of crude cabbage extract to linoleic and linolenic acid resulted in the formation of hexanal and *trans*-2-hexenal respectively as the end products (Fig 18), while these GLVs were not formed when linoleic and linolenic acid were directly subjected to radiation processing in vitro. This confirms the role of the enzymes in GLV formation. In their earlier work on the elucidation of mechanism of GLVs during wounding, Bate et al have also observed an enhanced liberation of GLVs without affecting enzyme activities [123]. They postulated that membrane damage due to wounding caused release of high content of free fatty acids that led to release of C6 volatiles without activation of enzymes of LOX pathway. In fact, in a study on Arabidopsis it was observed that, there was a little increase in GLVs in intact leaves of Arabidopsis even after over-expression of LOX and HPL [127]. Increased free fatty acid content is reported to be utterly essential to meet the demand for GLV formation during stress. Lipid radiolysis and consequent enhanced free fatty acid availability was noted in the radiation processed cabbage in the present study. Thus enhanced pool of free linolenic acid consequently formed, results in a greater substrate availability resulting in greater release of *trans*-hex-2-enal without activation of the enzymes of the LOX pathway.

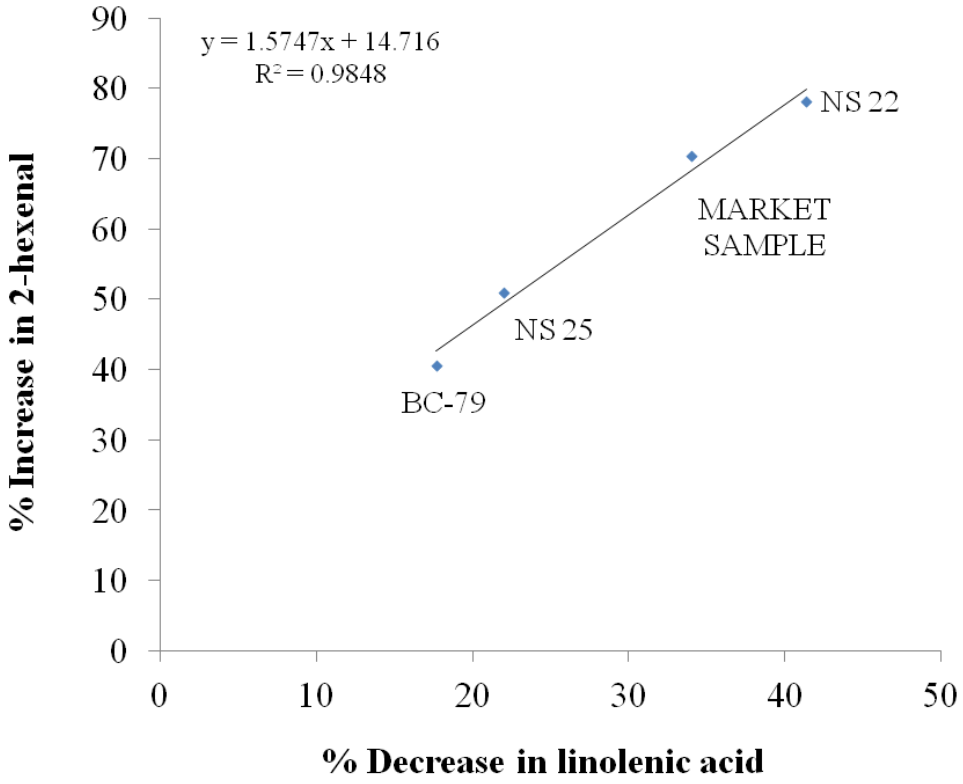


Fig 17 Plot depicting the relation between 2-Hexenal and linolenic acid content in irradiated cabbage.

Table 18 Activities (µmol/min/g of fw) of different enzymes of cabbage subjected to different radiation doses.

Enzyme	Control	0.5kGy	1kGy	2kGy
Lipase	0.02 ± 0.009^{a}	0.02 ± 0.01^{a}	0.0185 ± 0.009^{a}	0.021 ± 0.003^{a}
LOX	0.995 ± 0.08^{b}	0.96 ± 0.0213^{b}	0.88 ± 0.09^{b}	1.045 ± 0.16^{b}
HPL	1.32 ± 0.21^{c}	1.45 ± 0.33^{c}	1.44 ± 0.43^{c}	1.32 ± 0.48^{c}

Data are expressed as mean ± standard deviation (n=9). Mean values in the same row bearing different superscripts are significantly different ($p < 0.05$).

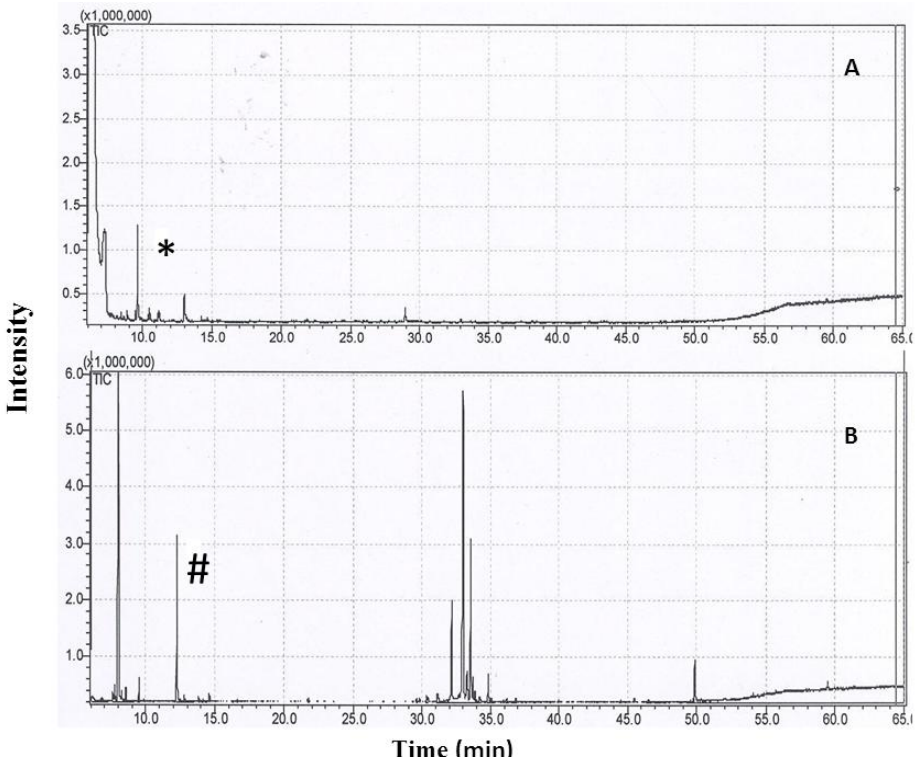


Fig 18 GCMS profile of products obtained by reaction of fatty acids substrate with crude enzyme extract of cabbage. A. Linoleic acid used as substrate B. Linolenic acid used as substrate

* Hexanal, # 2-Hexenal

3.3 Mechanism of AITC enhancement

Apart from GLV, an enhanced content of AITC was also observed in radiation processed cabbage. Due to its low threshold and higher concentration AITC is reported to be the key contributor to the characteristic cabbage aroma. AITC and other isothiocyanates in cabbage are reported to possess anticancerous property. Understanding the mechanism of radiation induced enhancement of these compounds can aid in designing strategies for improving nutraceutical value of the vegetable.

3.3.1 Estimation of glucosinolate content

Sinigrin is the glucosinolate precursor of AITC in cabbage. It is generally extracted in boiling water to prevent its enzymatic hydrolysis by inherent myrosinase during extraction. The aqueous extract thus obtained from different varieties of cabbage was further fractionated using ethyl acetate and n-butanol. TLC of these fractions (Fig 19) revealed the presence of glucosinolates mainly in the n-butanol extract. 2-Phenyl ethyl glucosinolate, 2-phenyl ethyl glucosinolate, p-hydroxy benzyl glucosinolate and sinigrin were the major glucosinolates identified by comparing their Rf values with available standards and from literature.

Since, n-butanol extract was mainly composed of glucosinolates this extract was subjected to HPLC analysis. Fig 20 A provides a representative HPLC profile of the glucosinolates present in the n-butanol extract of NS-22 cabbage variety. The extract was desulfated with sulfatase and further analyzed by HPLC. On desulfation these peaks were no longer detected confirming them to be glucosinolates (Fig 20 B). The major peak at Rt (11.6 min, Fig 20 A) was identified as sinigrin from its mass spectrum (m/z; 358 M^+) when subjected to LC/MS analysis and by comparison of its Rt with standard injected under similar condition. Table 19 provides the quantitative distribution of glucosinolates in different cabbage varieties. Sinigrin

was identified to be the major glucosinolate in all the three varieties studied. However, a wide variation in its content was noted with the highest in NS-22, followed by BC-79 and market samples. Variation in the content of this glucosinolate among different cabbage accessions ranging from 21.1- 4.3 μmol g^{-1} DW (dry weight) was reported by Kushad et al [124]. Song and Thornalley [94] have reported a sinigrin content of 5.09 ± 1.76 μmol/100 g in fresh green cabbage while its values were found to range from 41.0-28.2 μmol/100 g in fresh red cabbage [125]. The observed values of sinigrin content in the present study are in the range reported in literature. Differences in glucosinolate distribution pattern in *Brassica* have been observed between species and ecotype as well as between varieties and even within individual plants, depending on developmental stage, tissue and photoperiod [126]. As glucosinolates account for the distinctive flavours of cabbage, the wide variation observed in the sinigrin content between the different varieties can have a significant impact on their aroma and taste quality.

3.3.2 Effect of radiation processing on glucosinolate content

There is a limited understanding of the effects of post-harvest storage and processing on the glucosinolate content of *Brassica* vegetables. Refrigeration (4-8 °C), freezing, shredding and cooking in boiling water has been reported to significantly decrease glucosinolates in broccoli, brussel sprouts, cauliflower and green cabbage [94]. Jahangir et al [127] also reported a considerable decrease in the content of these compounds in vegetables such as broccoli, brussel sprouts, cauliflower and green cabbage when stored in domestic refrigerator (4-8 °C) for 7 days unlike when stored at ambient temperature. No changes in the content of glucosinolates were, however, noted when these vegetables were subjected to steaming, microwave cooking and stir-fry cooking. Oerlemans et al [128] have demonstrated a high thermal stability of glucosinolates during blanching (8%) compared to canning (75%) that

involves more drastic heat treatment. On the other hand an increase in indole and aliphatic glucosinolates was noted during controlled-atmosphere storage of broccoli for a period of 7

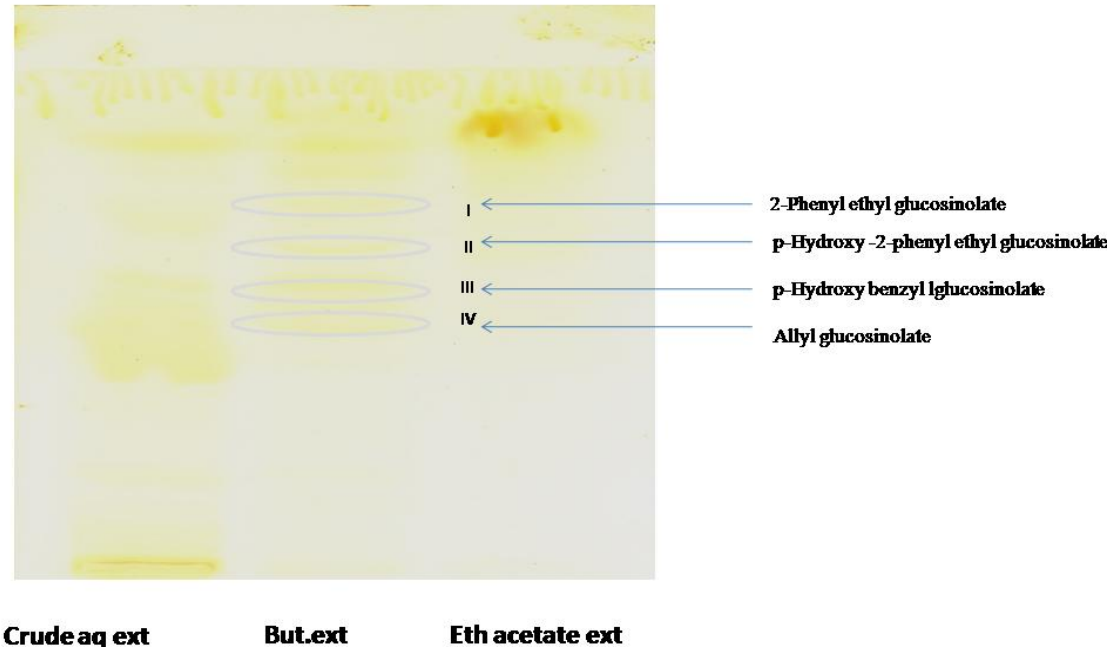


Fig19 TLC of glucosinolates

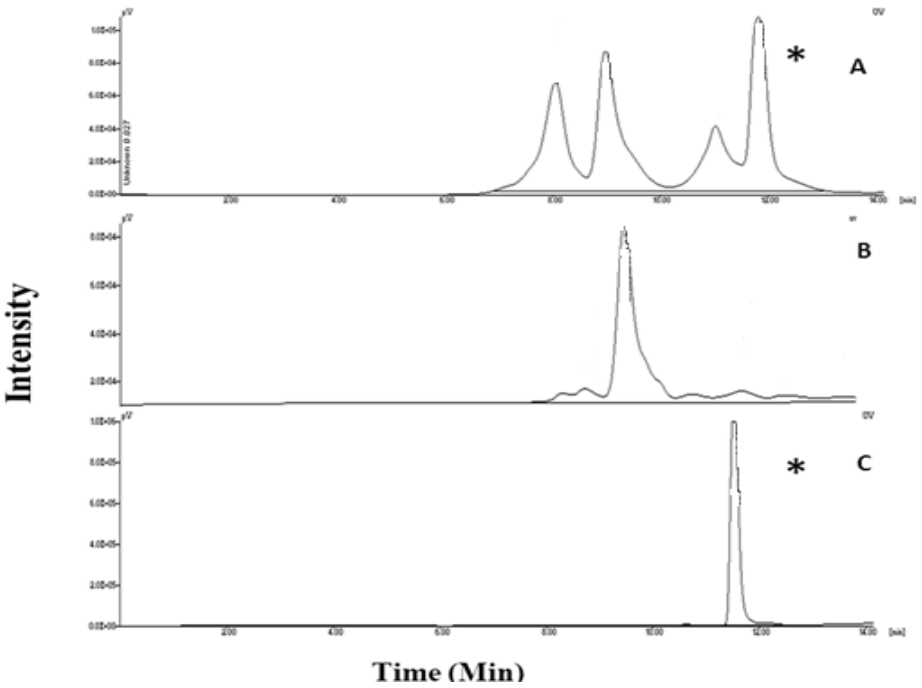


Fig 20 HPLC profile of aqueous cabbage extract. A. Aqueous extract. B. Aqueous extract treated with sulfatase enzyme. C. Sinigrin standard *= sinigrin

days at 7-13 °C. The varying results reported in literature suggest that the effect during storage can vary depending on the origin and variety of the vegetable as well as the processing history. The effect of post-harvest processing by ionizing radiations, a cold process, on the glucosinolate content have however, not been investigated so far. In the present study gamma irradiation was found to increase AITC content in irradiated cabbage thus indicating a change in the content of its precursor, Sinigrin. Since, maximum increase in AITC content was found at 2 kGy, the effect of radiation processing was studied in different cabbage varieties at this dose. Table 19 shows the effect of radiation processing (2kGy) on the sinigrin content on market sample, NS-22 and BC-79 variety of cabbage. An increase in the content of sinigrin was noted immediately after irradiation. The highest increase was noted in NS-22 (50%) followed by market samples (39%) and BC-79 (20%). The effect of radiation processing at three different doses of radiation on sinigrin content was further investigated. A linear increase was observed from 0.5 to 1kGy that remained constant beyond a dose of 1kGy (Fig 21A).

Apart from processing operations post harvest storage conditions of Brassica vegetables are also known to influence glucosinolate content. Content of these compounds was found to decrease in vegetables such as broccoli, brussel sprouts, cauliflower and green cabbage when stored in domestic refrigerator (4-8 °C) for 7 days in contrast to storage at ambient temperature [94]. Storage of vegetables at very low temperature (-85 °C) can result in freeze-thaw fracture of plant cells leading to significant loss of glucosinolates as a consequence of their conversion to isothiocyanates during thawing [94]. In the present work, cabbage leaves were irradiated and stored at 10 °C to prevent loss due to chill injury. The sinigrin content was estimated at different storage periods. The increase in sinigrin content was noted immediately after irradiation that remained constant on subsequent storage (Fig 21B).

Furthemore, a good correlation was also noted between increase in AITC and sinigrin content between different cabbage varieties (Fig 21C). Thus the increased AITC observed in the steam distilled volatile oils from radiation processed vegetable could be the result of hydrolytic breakdown of more available sinigrin in the treated samples.

A number of environmental conditions such as temperature, light, salinity plant nutritional status, fungal infection, wounding and insect damage can enhance glucosinolate content significantly [126]. In recent years, some researchers have demonstrated the effect of UV-B on glucosinolate metabolism. A study on the effect of UV radiation on *Tropaeolum majus* demonstrated that low UV-B treatment induced a six fold increase in benzyl glucosinolates [129]. Wang et al [130] showed that UV-B radiation induced production of several glucosinolates in *A thaliana*. Continuous UV-B exposure, however, inhibited the expression of glucosinolate metabolism related genes resulting in a significant decline in glucosinolate content, particularly that of indolic glucosinolates. Mewis et al [93] have recently reported an increase in aliphatic glucosinolates in broccoli sprouts on exposure to UV-B radiation. They demonstrated that this increase on exposure to UV-B was a result of up-regulation of genes involved in glucosinolate biosynthesis. A similar effect at genetic level could possibly account for the enhanced sinigrin observed in the present study.

3.3.3 Effect of radiation processing on myrosinase activity

Tissue damage as result of postharvest processing of the vegetable can result in cellular breakdown and a consequent hydrolysis of glucosinolates by endogenous myrosinase. The presence of active myrosinase is a prerequisite for formation of bioactive breakdown products of glucosinolates such as isothiocyanates that in turn determine their final intake levels. There are however very few reports on the effect of postharvest processing on myrosinase activity. Earlier work has reported a loss of myrosinase activity in cabbage after 2 min of

microwave cooking and after 7 min of steaming [131]. Dekker and Verkerk [125] have also demonstrated a diminished myrosinase activity with increasing input of microwave energy. Similar trends have also been shown under increasing pressure [132]. In the present study the enhanced AITC observed in the essential oils of irradiated vegetable suggests either an increased myrosinase activity or a greater enzyme substrate interaction as a consequence of increased sinigrin availability. No significant change in the myrosinase activity was observed as a result of radiation processing at doses of 0.5-2 kGy in the present study (Fig 22). In an earlier work, Lessman et al [133] have reported inactivation of myrosinase without degradation of glucosinolates when mustard and rape were exposed to gamma radiation dose of 5 kGy. Lower doses presently employed may possibly account for the ineffectiveness of radiation treatment in affecting myrosinase activity. *In vitro* exposure of sinigrin in aqueous solution to radiation processing further ruled out the formation of volatile AITC by direct radiolysis of sinigrin. Thus retention of myrosinase activity can aid in greater availability of bioactive degradation products from the increased sinigrin formed as a consequence of radiation processing and thus enhance their final intake levels.

Table 19 Effect of irradiation (2 kGy) on glucosinolate content (µmol/100g of fw) in 3 varieties of cabbage.

VARIETY	NS-22		BC-79		Market sample	
	Control	Irradiated	Control	Irradiated	Control	Irradiated
Sinigrin	58.15 ± 2.7[a]	87.23 ± 5.08[b]	35.8 ± 2.2[a]	42.96 ± 4.98[b]	24.1 ± 2.8[a]	33.5± 3.9[b]
Progoitrin	38.15 ± 1.7[a]	38.15 ± 1.7[a]	21.15 ± 1.7[a]	21.15 ± 1.5[a]	24.15 ± 1.7[a]	21.15 ± 0.7[a]
Gluconastrulin	18.15 ± 2.1[a]	38.15 ± 1.7[a]	10.15 ± 2.1[a]	9.15 ± 1.1[a]	11.15 ± 2.2[a]	8.15 ± 2.1[a]

Data are expressed as mean ± standard deviation (n=9). Mean values in the same column for a variety bearing different superscripts are significantly different ($p < 0.05$).

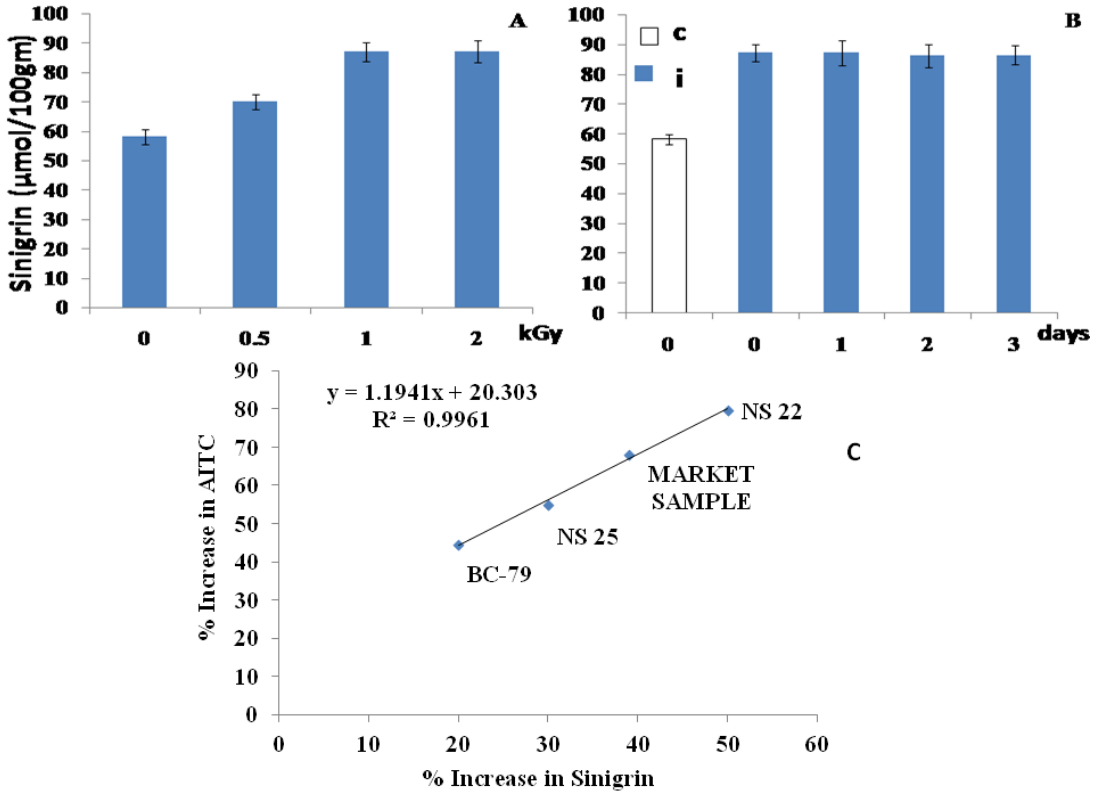


Fig 21 A) Effect of radiation dose on sinigrin content. B) Effect of storage after irradiation (2kGy) on sinigrin content. C) Plot depicting the relation between AITC and sinigrin content in irradiated cabbage. Values are expressed as mean ± SD (n=9). c- Control, i- Irradiated

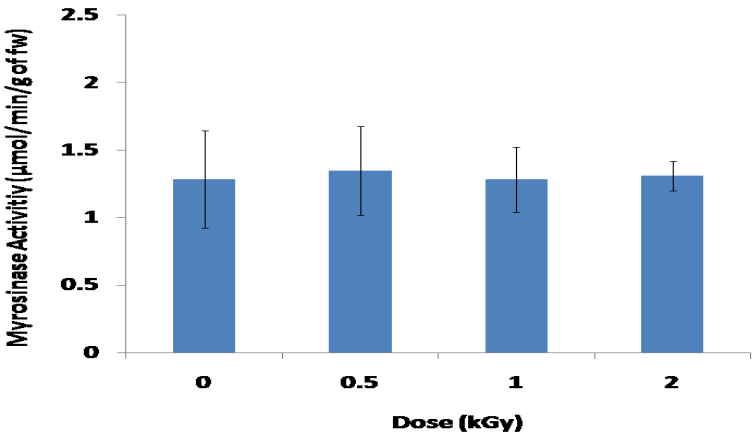


Fig 22 Myrosinase activity (µmol/min/g of fw) of cabbage (NS-22) subjected to different radiation doses.

Enhancement of sinigrin, the major glucosinolate of cabbage, due to radiation processing was thus responsible for AITC enhancement in irradiated sample. Glucosinolates are known to be regulated both developmentally and environmentally in various organs and tissues depending on the type of biotic and abiotic stresses. Substantial work has been carried out on the effect of various stress factors such as temperature fluctuation, drought, UV irradiation, herbivore and pathogen attack on glucosinolate biosynthesis in brassica species. However, the effect of gamma irradiation on glucosinolate biosynthesis has not been investigated.

3.3.4 Effect of gamma irradiation on jasmonate content

Selective induction of glucosinolates on exposure to jasmonates has been widely reported. In many cases jasmonates are reported to be the key signaling molecule inducing the expression of glucosinolate biosynthesis genes that activate glucosinolate biosynthesis. The jasmonates are the byproducts of lipoxygenase pathway of membrane lipid metabolism. Linolenic acid produced from lysis of membrane lipids produce jasmonic acid by the action of enzyme allene oxide synthase. Since in the present study radiation induced enhancement in linolenic

acid content was observed hence a correlation between gamma irradiation induced increase in linolenic acid, formation of jasmonic acid and glucosinolate enhancement was speculated.

Jasmonic acid, a 12-carbon fatty acid cyclo-pentanone (and/or its precursors), its conjugates and precursors, are collectively referred to as jasmonates (JAs). The jasmonate family is defined as biologically active cyclopentenones and cyclopentanones of related structure originating from the octadecanoid and hexadecanoid biosynthetic pathways. Jasmonates are known to regulate the biosynthesis of a number of secondary metabolites including glucosinolates. However, the exact mechanism of this regulation is not elucidated. Moreover, the nature of glucosinolates which are induced by the jasmonates remains questionable. Some researchers have reported the induction of aliphatic glucosinolate by exogenous application of jasmonic acid while others have reported the same for indolyl glucosinolates. No reports however have dealt with the effect of gamma irradiation on biosynthesis of glucosinolate or jasmonic acid.

In majority of studies on the effect stress on jasmonate level, the expression of jasmonate biosynthetic genes have been analyzed. However, the endogenous levels of bioactive hormones are not necessarily reflected by the expression levels of the corresponding biosynthesis genes [134]. Hence, in the present study the jasmonic acid and methyl jasmonate content was quantified in the cabbage samples before and after irradiation. These compounds were extracted using SPE column. This method is known to be highly selective and sensitive for these compounds. Control as well as irradiated samples (0.5-2 kGy) was extracted immediately after irradiation. Fig 23 shows the HPTLC chromatograph of jasmonates. Quantification was done using densitometric analysis of samples and standards of methyl jasmonate and jasmonic acid. No change in jasmonate content was noted between control and irradiated samples immediately after irradiation at all the doses studied. A lag period is

normally present between the time plant tissue encounters stress and production of signalling molecules like jasmonates in plant tissues. Engelbirth et al reported maximum induction of jasmonates after 1 hr of injury [134]. The jasmonate content in irradiated cabbage was therefore analyzed at different time points (0 hr, 0.5 hr, 1hr and 24 hr after irradiation). No change in jasmonate content was found at any of the time points (Table 20). Radiation processing was thus found to have no effect on jasmonate content. Hence, jasmonates are not likely to act as signaling molecules for radiation induced glucosinolate enhancement in irradiated cabbage.

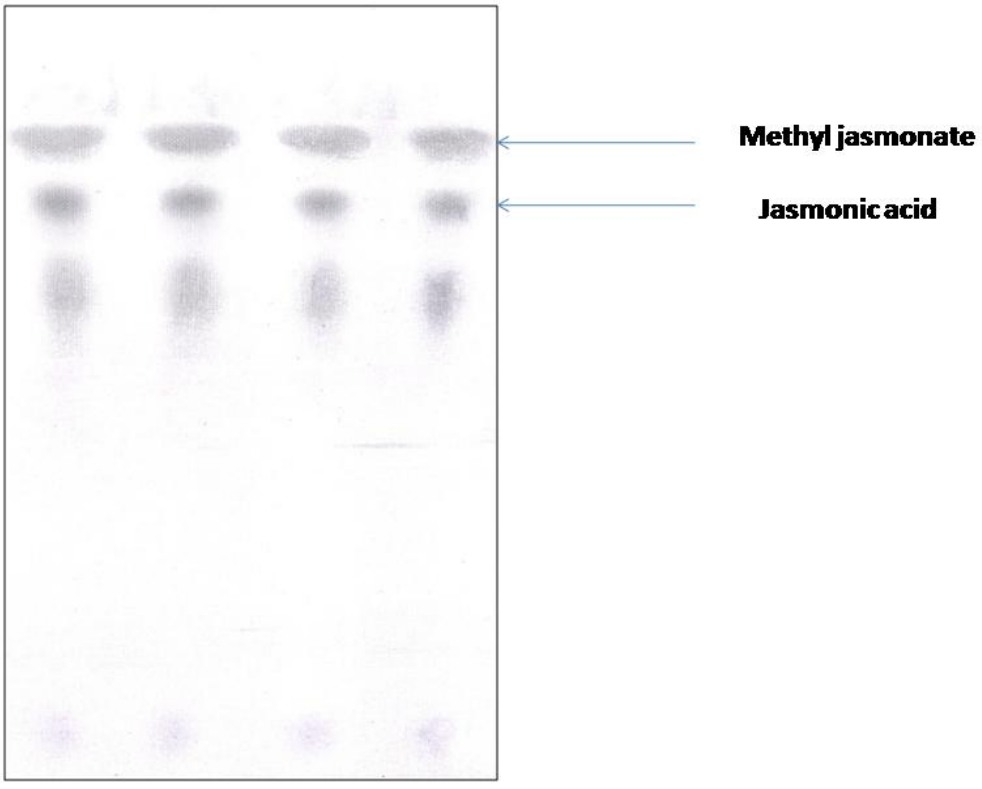


Fig 23 HPTLC of jasmonates

Table 20 Effect of gamma irradiation on jasmonates in cabbage

Dose (kGy)	Time (hr)	Jasmonic acid (µg/kg FW)	Methyl Jasmonate (µg/kg FW)
Control	0	0.42 ± 0.08^{a}	0.98 ± 0.08^{b}
	0.5	0.43 ± 0.18^{a}	1.02 ± 0.18^{b}
	1	0.45 ± 0.27^{a}	0.95 ± 0.27^{b}
	24	0.41 ± 0.11^{a}	0.97 ± 0.11^{b}
0.5 kGy	0	0.38 ± 0.21^{a}	1.01 ± 0.21^{b}
	0.5	0.41 ± 0.18^{a}	0.82 ± 0.18^{b}
	1	0.37 ± 0.11^{a}	1.04 ± 2.01^{b}
	24	0.37 ± 0.11^{a}	1.02 ± 0.28^{b}
1 kGy	0	0.45 ± 0.18^{a}	0.95 ± 0.18^{b}
	0.5	0.48 ± 0.09^{a}	0.98 ± 0.09^{b}
	1	0.41 ± 0.28^{a}	1.02 ± 0.28^{b}
	24	0.41 ± 0.08^{a}	0.91 ± 0.08^{b}
2 kGy	0	0.37 ± 0.19^{a}	0.92 ± 0.19^{b}
	0.5	0.42 ± 0.18^{a}	1.09 ± 0.18^{b}
	1	0.42 ± 0.18^{a}	1.02 ± 1.09^{b}
	24	0.38 ± 0.08^{a}	0.98 ± 0.08^{b}

Data are expressed as mean ± standard deviation (n=9). Mean values in the same column for a variety bearing different superscripts are significantly different ($p < 0.05$).

3.3.5 Effect of gamma irradiation on glucosinolate synthesis genes

Glucosinolate biosynthesis in brassica vegetables is known to be genetically regulated by a cascade of genes. These genes are induced by a variety of biotic and abiotic stresses resulting in a variation in the glucosinolate content [85]. The effect of gamma irradiation on the expression of these genes has however, not been reported so far. The effect of radiation processing on the expression of genes involved in glucosinolate biosynthesis was further explored.

Biosynthesis of GSLs can be divided into a) chain elongation of selected precursor amino acids (mainly methionine) by addition of methylene groups b) formation of core glucosinolate structure by reconfiguration of the amino acid moiety and c) secondary modification of the amino acid side chain by hydroxylations, methylations, oxidations or de-saturations. The subsequent steps are regulated by a battery of genes which act sequentially to form the individual GSLs. The aliphatic GSL composition in *A. thaliana* ecotypes and Brassica species is highly variable and considered to be under strong genetic control. The expression levels of seven genes in glucosinolate biosynthetic pathway were determined, including the transcription factor (*MYB28*) the structural genes encoding enzymes of aliphatic glucosinolate biosynthesis (*CYP79F1* and *CYP83A1*), genes involved in both aliphatic and indolic (*SUR1, UGT74B1 AND SOT18*) and the genes encoding enzymes for GSL degradation (*TGG1*).

The change in expression of the glucosinolate biosynthesis genes due to gamma irradiation (2 kGy) is shown in Fig 24a. A number of genes were found to be upregulated in response to radiation processing including *MYB 28, CYP79F1, CYP83A1, SUR1* and *UGT74B1*. *MYB28* expression doubled in irradiated cabbage. MYB transcription factors are important components in the complex signalling pathways of plant defense against abiotic stress like extreme temperature, nutrient deficiencies etc. It represents a key component in the regulation

of aliphatic methionine-derived GSL biosynthesis in *A. thaliana*. Kim et al., demonstrated a correlation between the content of aliphatic GSLs and gene expression level of *MYB28* in Chinese cabbage. MYB promoters were found to regulate expression of the genes under osmotic stresses in *A. thaliana* [87]. Hirai et al. investigated *MYB28* and *MYB29* as master transcription factors of GSL biosynthesis; *MYB29* has a role in JA-mediated aliphatic glucosinolate biosynthesis [135]. However, role of JA in induction of *MYB28* has not been reported so far. In the present case gamma irradiation was not found to bring about any change in endogenous JA content in the irradiated samples. This could very well justify the induction of *MYB28* in the irradiated samples. *MYB28* regulates the pathway from methionine to aliphatic glucosinolates, and is essential for the basal-level control of aliphatic glucosinolate biosynthesis [135]. Hence, upregulation of this gene could be the reason behind enhancement in sinigrin content in irradiated vegetable.

The structural genes i.e. *CYP79F1* and *CYP83A1* were also up-regulated by gamma irradiation. Cytochrome P450 monooxygenases are a group of haem-containing proteins which catalyze various oxidative reactions. In higher plants, cytochrome P450s play crucial roles in biosynthesis of a variety of endogenous lipophilic compounds such as fatty acids, sterols, phenylpropanoids, terpenoids, phytoalexins, brassinolides and gibberellins. In addition, oxidative detoxification of a number of herbicides in plant tissues is also achieved by a cytochrome P450- dependent monooxygenase system [136,137]. Despite these important roles, little is known about their induction patterns in response to environmental stimuli, especially to abiotic stress or a pathogen challenge. These two genes form the most important genes for core structure synthesis. *CYP79F1* converts all chain-elongated Met derivatives to the respective oximes. The aliphatic aldoximes are converted by *CYP83A1* to form the 'activated oxime'. In an earlier work by Mewis et al [93], the Brassica microarray data

revealed that UV-B increased the expression of genes presumably coding for families of Cyt P450 monooxygenase leading to increase in glucosinolate content by 2-folds. In another study it was reported that insect feeding on Arabidopsis thaliana led to changes in transcript levels of some genes of GS biosynthesis [85]. There were significant increases in expression levels of genes of aliphatic GS biosynthesis, such *CYP79F1* but genes of indolyl GS biosynthesis, such as *CYP79B2, CYP79B3*, and *CYP83B1*, were generally not affected. These results correspond to the increases in aliphatic GS content observed after feeding by these three insect species [85]. *MYB28* is the transcriptional factor regulating the expression of *CYP79F1* and *CYP83A1* genes [85]. Hence, in the present study the gamma irradiation induced increase in *MYB28* gene expression could lead to an increase in expression of *CYP79F1* and *CYP83A1*.

Following conjugation of the activated aldoximes to a sulfur donor which can happen non-enzymatically, the produced S-alkyl-thiohydroximates are converted to thiohydroximates by the *SUR1*. A very small up-regulation was seen in *SUR1* due to gamma irradiation. Thiohydroximates are in turn S-glucosylated by glucosyltransferases of the UGT74 family to form desulfoglucosinolates. Gamma irradiation was found to slightly up-regulate *UGT74B1*. The glucosylation gives rise to desulfoglucosinolates, which are finally sulfated by the sulfotransferases *SOT18* to form glucosinolates. No significant difference was seen in SOT18 due to gamma irradiation. While these three genes are necessary for GSL biosynthesis, change in their expression pattern is of secondary importance. The change in aliphatic glucosinolate content has been primarily correlated with *MYB28, CYP79F1* and *CYP81A1* by different authors and a change in expression of these genes has been recognized as being the rate limiting steps by different authors in glucosinolate synthesis. Hence, enhancement in

glucosinolate content due to gamma irradiation in cabbage could be very well attributed to enhancement in glucosinolate biosynthesis genes.

The glucosinolates in cells are hydrolyzed by the enzyme myrosinase. Hence, an increase myrosinase activity could result in decrease in the content of glucosinolates. Our studies on myrosinase activity have demonstrated constant myrosinase activity in irradiated cabbage. Transcriptional analysis of *TGG1*, gene encoding for myrosinase enzyme further confirmed this finding. No significant change in *TGG1* gene expression was noted in irradiated samples.

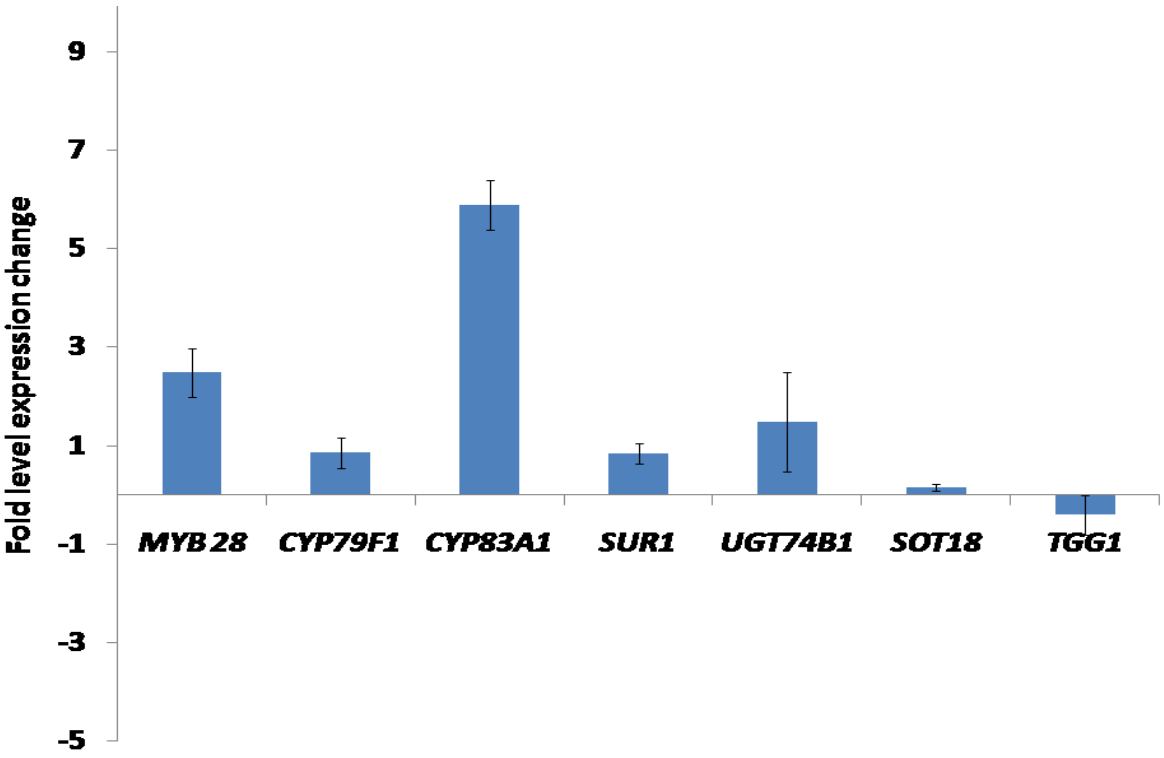


Fig 24a Fold change in the expression of different glucosinolate biosynthetic genes in irradiated sample (2kGy). The x-axis represents the expression level of genes in control sample. All values are means of triplicates ±SD.

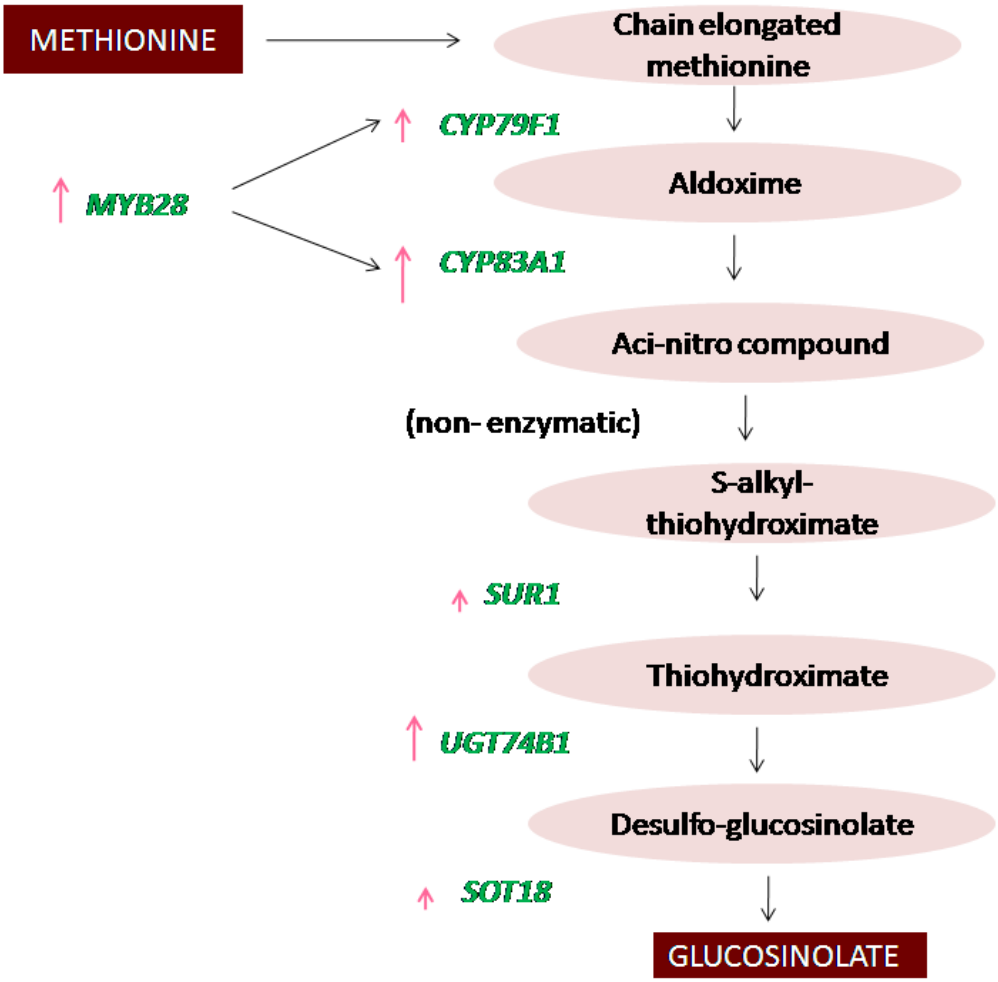


Fig 24b Proposed mechanism of gamma irradiation induced glucosinolate enhancement at genetic level in cabbage.

3.4 Development of minimally processed ready to eat shredded cabbage using radiation processing

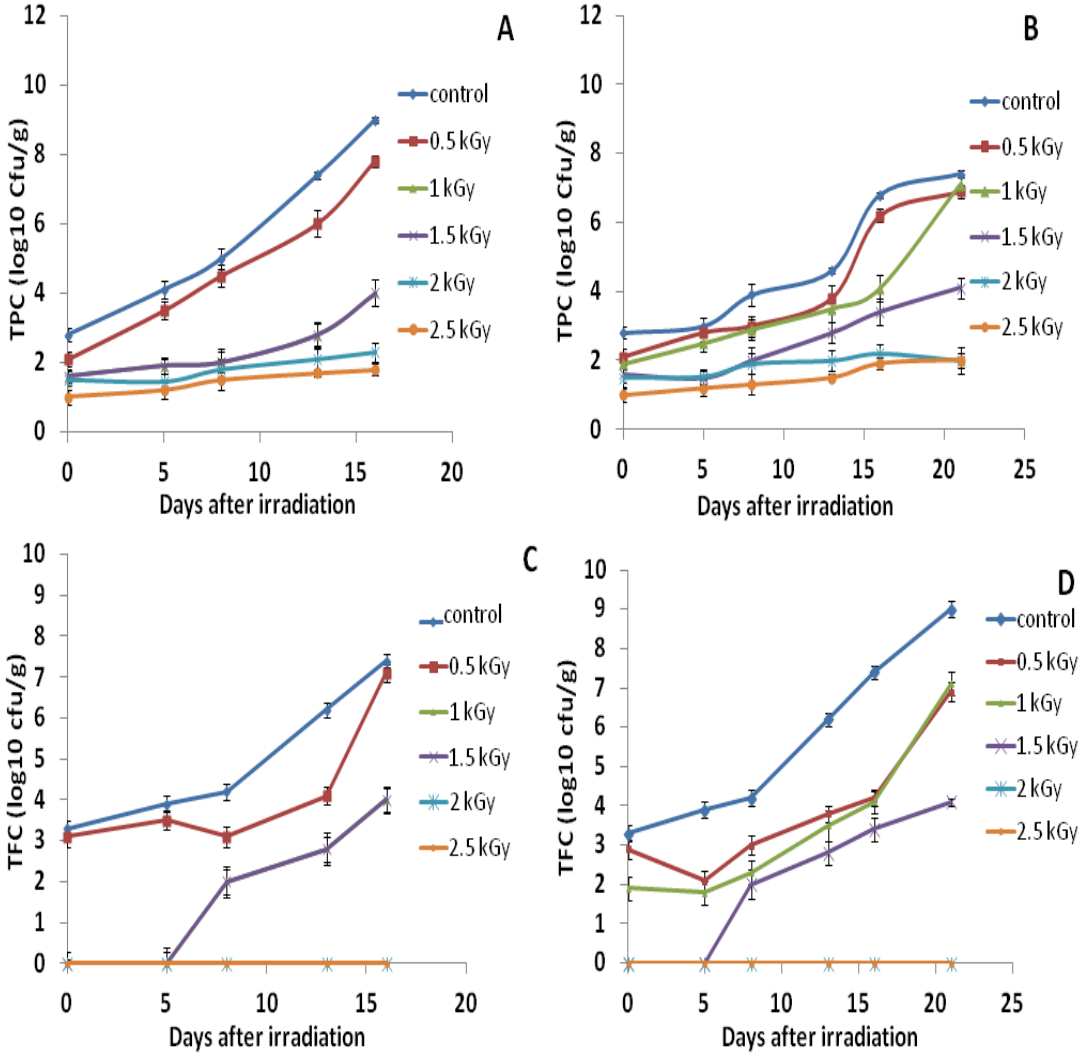
Cabbage and other Brassica vegetables are widely marketed as a minimally processed product in the shredded form due to the associated convenience and fresh characteristics. Use of radiation processing for microbial decontamination of minimally processed cut vegetables and fruits for increased shelf life have been widely reported [51]. In our studies so far, gamma irradiation was found to improve the sensory quality of cabbage by improving its aroma quality. Due to the increasing importance of minimally processed shredded cabbage for preparation of a wide variety of recipes including salad preparations, it was of interest to determine the feasibility of using radiation processing for improving its sensory properties in addition to enhanced shelf life.

Ahn et al demonstrated the use of modified atmosphere packaging and gamma irradiation for preservation of cut salted Chinese cabbage at 4°C [138]. Several countries recommend a temperature of 0 ºC for storage of minimally processed vegetables. However, during shipment and marketing temperatures normally reach 5-10 °C resulting in their lower acceptability [50]. In India, minimally processed vegetables are generally stored at 10 ºC in supermarket that can result in substantial losses [139]. About one third of the horticultural produce in developing countries is known to be lost due to poor cold storage facility [140]. Thus preservation methods that aid in safe storage at higher temperature is preferred. However, majority of the work carried out on gamma irradiation of minimally processed products involves storage at 0-4 °C. Potential use of gamma irradiation in enhancing shelf life of minimally processed fruits and vegetables at higher temperature therefore needs to be further explored.

An extensive review on the use of gamma irradiation for shelf life extension of different vegetables has been recently published by Arvanitoyannis [51]. The optimum dose for shelf life extension of Brassica vegetables lies between 1-2 kGy. Hence in the present study gamma irradiation (0.5-2.5 kGy) in combination with low temperature (4-15 °C) storage was attempted to increase shelf life of ready-to-eat shredded cabbage. Fresh shredded cabbage samples were processed as reported in experimental section 2.8. At 15 ºC rapid microbial and physiological spoilage resulted in a very short shelf life for both control and irradiated samples. Hence samples stored at lower temperatures (4 and 10 ºC) were used for further experiment. Overall quality of the samples with respect to microbial, sensory and nutritional parameters was evaluated at different periods of storage.

3.4.1 Microbial analysis

Gamma irradiation is known to be an effective tool for reducing both bacterial and fungal population. Effect of irradiation (0.5 to 2.5 kGy) on bacterial load is shown in Fig 25. Irradiation was found to decrease the bacterial load at all doses studied. In the present study a dose dependent decrease in bacterial and fungal population was noted. Increasing radiation dose resulted in a decrease in bacterial load with a reduction by 2 log cycles at both 2.0 & 2.5 kGy. A significant ($p \leq 0.05$) increase in bacterial load during storage was observed in the non-irradiated control samples. In the control samples stored at 10 and 4 °C, the counts reached higher than 10^7CFU/g on day 13 and 16 respectively which is beyond the acceptable limit (10^7CFU/g) prescribed for fresh cut vegetables and fruits (Fig. 25A & 25B). A similar trend was seen in samples irradiated at 0.5 kGy for both the temperatures. Between 1.0-1.5 kGy the above acceptable limit of bacterial population was reached within 16 days and 21 days at 10 and 4 °C respectively. However, at radiation doses of 2 and 2.5 kGy the mesophillic counts were well below the acceptable limits up to 16 and 21 days when stored at 10 and 4 °C.



Fig

Fig 25 Total plate count (TPC) of RTC cabbage during storage at **A**. 10 ºC, **B**. 4ºC; Total fungal count (TFC) count of RTC cabbage during storage at **C**. 10ºC; **D**. 4ºC. Values are expressed as mean ± SD (n=9). Samples stored at 10 °C and 4 °C were studied for 16 days and 21 days respectivly

Results obtained in the present study are in agreement with that reported previously by other researchers.

The response of yeast and mould count for different radiation doses is shown in Fig 25 C & 25 D. Control samples showed an increase in fungal count with storage. No fungi were detected in cabbage irradiated with a dose of 1.5 kGy until 5 days of storage. In samples irradiated at doses ≥ 2kGy no fungi were detected during the entire storage period of 16 and 21 days at 10 and 4 ^{0}C respectively. Similar results have been reported for irradiated lettuce [141] and other vegetables [142] . Khattak et al [143] found that fungal colonies were eliminated on carrot and cabbage when treated with doses higher than 2kGy. It has been proposed that elimination of spoilage bacteria can bring about enhanced growth of pathogenic bacteria. A reduction by 4-5 log cycles in the population of *Listeria monocytogenes* in radiation processed leafy vegetables such as water cress has been well documented [104]. Therefore pathogenic microorganisms were not monitored in the present study.

Thus based on microbial analysis an optimum shelf life of 16 and 21 days could be achieved when shredded cabbage was radiation processed at doses ≥ 2kGy and stored at 10 and 4 ^{0}C respectively. Thus, 2 kGy was found to be the optimum dose required for maintenance of microbial safety during storage of RTC cabbage.

3.4.2 Sensory analysis

3.4.2.1 Hedonic testing

Sensory attributes of RTC cabbage at different irradiation doses and storage time based on hedonic analysis is given in Table 21and 22. Sensory quality of control sample stored at 10 °C was found to deteriorate within 5 days due to browning of the cut edges with a higher browning intensity on day 8. This was also the case with the control samples stored at 4 °C beyond 13 days of storage. Samples irradiated with doses from 0.5 to 1.5 kGy also showed

significant blackening at the end of storage period at both the temperatures. However, 2 and 2.5 kGy appeared fresh throughout the storage period. The aroma quality of control sample and samples subjected to doses less than 2 kGy decreased with storage. Off odor was detected by the panel in the control, 0.5 and 1 kGy samples at the end of the storage period for both the temperatures. No off odor was, however, detected in 2 and 2.5 kGy sample throughout the storage period. In fact an increase in cabbage like odor was noted in these samples that remained unaffected with storage. The 2 kGy sample showed a slight softening in texture immediately after irradiation. However, this did not affect the overall sensory acceptability of the sample. The control sample as well as the 0.5 to 1.5 kGy treated samples showed a decrease in firmness in later stages of storage while the 2 kGy sample maintained its texture throughout the storage period. The 2.5 kGy irradiated sample, however, showed a considerable softening in texture at all storage intervals which reduced overall acceptability of these samples. No difference in taste was found between control and irradiated sample immediately after irradiation. At the end of storage period control sample and samples given a dose of less than 2 kGy were extensively spoiled due to microbial load and hence could not be assessed by the sensory panel for taste at later stages of storage. The 2 and 2.5 kGy samples showed a slight decrease in taste score at the end of storage period but it was found to be within the acceptable limit.

Thus based on microbial and sensory analysis at the optimum conditions of irradiation (2 kGy) and storage at 10 and 4 °C an extension in shelf life by 8 and 5 days respectively was achieved compared to the non-irradiated controls.

3.4.2.2 Color determination

As 2 kGy was found to be the optimum dose from sensory and microbial analysis, further analyses were carried out on the samples irradiated at this dose. Figure 26A & 26B represents the effect of irradiation and storage on L values of RTC cabbage strips. A continuous decrease in L values during storage, with a substantial decrease beyond day 5 at 10°C and 13 days at 4 °C was seen in control sample. Ke and Saltveit [144] have reported similar observations in fresh-cut vegetables. Interestingly, luminosity (L) of the cabbage strips irradiated at 2 kGy remained unchanged during storage and the visual quality was acceptable at the end of storage. Polyphenoloxidase (PPO), that catalyses the oxidation of phenolic compounds to colored melanins, is the major enzyme responsible for browning in fruits and vegetables. In an earlier report, a five-fold increase in alpha resorcylic acid, an inhibitor of PPO, in irradiated minimally processed cut ash gourd cubes resulting in prevention of browning in this product was demonstrated. Lowering in PPO activity as a consequence of conformational changes in the active site on exposure to radiation and a resulting browning inhibition in irradiated mushroom has been reported. Changes in PAL activity under stress and a consequent variation in content of various benzoic and cinnamic acid derivatives that are known to be either substrates or inhibitors of PPO have also been reported. Further work on the mechanism of browning inhibition in cabbage by gamma irradiation in the light of the above observations is detailed in later section.

Table 21 Effect of irradiation dose on different sensory parameters of RTC cabbage stored at 10 °C.

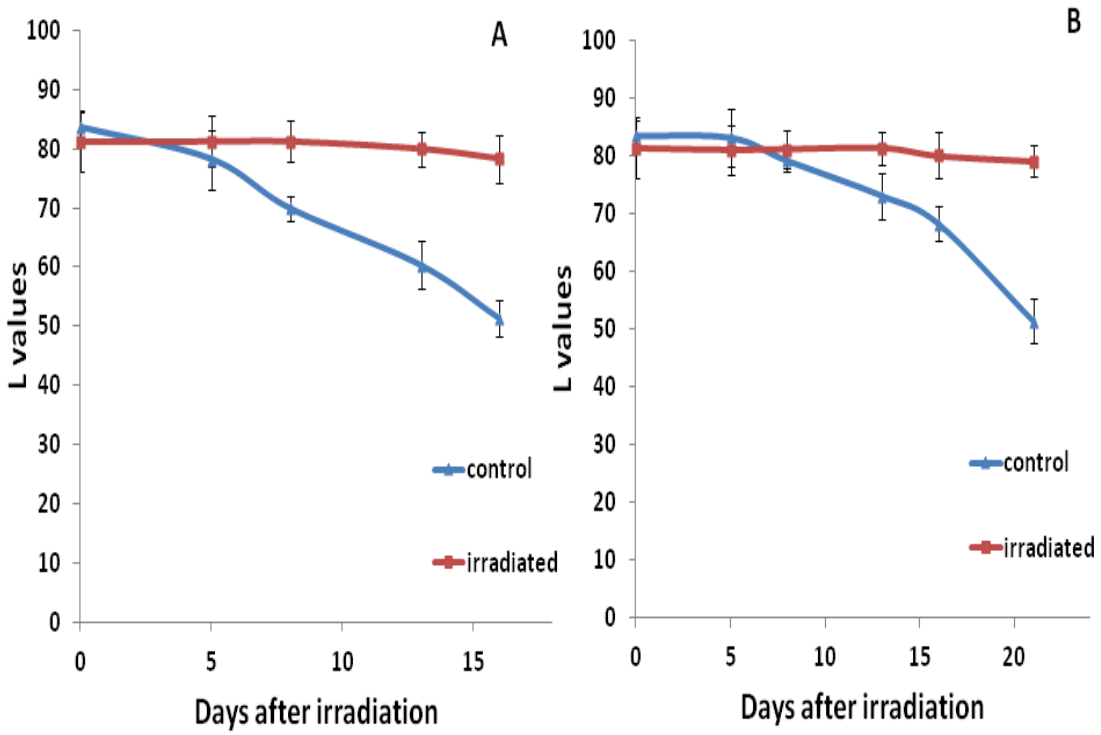
Day	Dose (kGy)	Color	Texture	Taste	Aroma	Over all acceptabilityy
0	0	7.1±1.1^{a}	7.1± 1.1^{a}	6.9±2.2^{a}	6.8±0.4^{b}	7.1±1.1^{a}
	0.5	7.1±1.8^{a}	7.2±1.2^{a}	7.1±1.2^{a}	6.3±0.2^{b}	7.1±1.2^{a}
	1	7.9±1.1^{a}	7.7±1.1^{a}	6.4±2.2^{a}	6.2±0.7^{b}	7.9±1.1^{a}
	1.5	7.1±1.5^{a}	7.1±1.5^{a}	6.1±1.2^{a}	5.9±0.5^{b}	7.1±1.2^{a}
	2	7.3±1.2^{a}	6.1±1.1^{a}	6.9±2.1^{a}	8.3±0.2^{a}	7.3±1.2^{a}
	2.5	7.2±1.1^{a}	4.2±0.4^{b}	7.1±1.3^{a}	8.7±0.8^{a}	5.1±0.3^{b}
5	0	5.3±0.1^{b}	7.1±1.1^{a}	6.1±2.1^{a}	5.6±1.2^{b}	5.1±0.6^{b}
	0.5	6.8± 1.2^{a}	7.2±1.2^{a}	7.2±1.1^{a}	5.2±0.8^{b}	6.8±.1.2^{a}
	1	7.2±1.5^{a}	6.8±2.1^{a}	6.4±2.2^{a}	5.3±0.4^{b}	7.2±1.5^{a}
	1.5	7.9±1.1^{a}	6.2±1.6^{a}	6.1±1.8^{a}	6.1±1.1^{ab}	7.9±1.1^{a}
	2	7.1±1.8^{a}	5.7±1.2^{a}	6.1±2.3^{a}	8.1±0.2^{a}	7.1±1.8^{a}
	2.5	7. 2±1.5^{a}	4.2±0.9^{b}	7.1±1.3^{a}	8.1±0.1^{a}	5. 2±0.5^{b}
8	0	3.4±1.1^{b}	6.4±2.7^{a}	5.5±2.2^{a}	4.1±0.8^{c}	3.4±1.1^{c}
	0.5	4.1±1.1^{b}	6.8±2.1^{a}	6.2±1.6^{a}	4.3±1.1^{bc}	5.1±0.4^{b}
	1	7.2±0.3^{a}	7.1±1.2^{a}	7.2±1.8^{a}	5.1±1.4^{b}	5.2±0.3^{b}
	1.5	7.6±1.4^{a}	6.6±1.4^{a}	6.2±1.9^{a}	5.8±0.8^{b}	6.6±0.4^{a}
	2	7.9±1.1^{a}	5.5±2.2^{a}	6.2±1.9^{a}	7.7±1.3^{a}	7.1±0.8^{a}
	2.5	7.7±1.3^{a}	4.2±0.5^{b}	7.1±1.2^{a}	7.2±1.5^{a}	5.1±0.3^{b}
13	0	2.1±0.9^{c}	4.3±0.1^{b}	NA	3.1±0.1^{c}	2.1±0.9^{c}
	0.5	2.5±1.2^{c}	4.1±0.3^{b}	NA	3.1±0.1^{c}	2.5±0.2^{c}
	1	5.2±2.3^{a}	6.1±0.4^{a}	5.2±2.4^{a}	4.2±0.2^{b}	4.2±1.3^{b}
	1.5	6.6±1.2^{a}	6.1±0.3^{a}	5.6±2.1^{a}	4.1±0.2^{b}	6.6±1.2^{a}
	2	7.4±1.8^{a}	6.5±0.5^{a}	6.8±1.6^{a}	7.8±1.2^{a}	7.4±1.8^{a}
	2.5	7.1±1.1^{a}	4.8±0.6^{b}	7.2±3.8^{a}	7.4±2.3^{a}	5.1±1.1^{b}
16	0	2.1±1.1^{c}	4.1±1.1^{b}	NA	2.1±2.1^{c}	2.1±0.6^{c}
	0.5	2.2±0.5^{c}	4.2±0.1^{b}	NA	2.6±2.3^{c}	2.2±0.5^{c}
	1	3.1±1.1^{c}	4.3±0.2^{b}	NA	3.4±1.8^{c}	3.1±0.5^{c}
	1.5	5.2±2.3^{a}	5.3±1.2^{b}	NA	4.1±0.8^{b}	4.2±0.3^{b}
	2	7.8±1.2^{a}	6.9±1.3^{a}	6.1±1.2^{a}	6.1±1.4^{a}	7.8±1.2^{a}
	2.5	6.1±2.1^{a}	4.4±1.4^{b}	5.9 ±1.2^{a}	6.2±1.2^{a}	5.1±1.1^{b}

Data are expressed as mean ± standard deviation (n=9). Mean values in the same row bearing different superscripts are significantly different ($p < 0.05$).

Table 22 Effect of irradiation dose on different sensory parameters of RTC cabbage stored at 4 ºC.

Day	Dose (kGy)	Color	Texture	Taste	Aroma	Over all acceptability
0	0	7.1±1.1^{a}	7.1± 1.1^{a}	6.9±1.2^{a}	6.8±0.4^{b}	7.1±1.1^{a}
	0.5	7.1±1.8^{a}	7.2±1.2^{a}	7.1±1.2^{a}	6.3±0.2^{a}	7.1±1.2^{a}
	1	7.9±1.1^{a}	7.7±1.1^{a}	6.4±2.2^{a}	6.2±0.7^{b}	7.9±1.1^{a}
	1.5	7.1±1.5^{a}	7.1±1.5^{a}	6.1±1.2^{a}	5.9±0.5^{b}	7.1±1.2^{a}
	2	7.3±1.2^{a}	6.1±1.1^{a}	6.9±2.1^{a}	8.3±0.2^{a}	7.3±1.2^{a}
	2.5	7.2±1.1^{a}	4.2±0.4^{b}	7.1±1.3^{a}	8.7±0.8^{a}	5.1±0.3^{b}
5	0	6.3±1.6^{a}	7.2±1.1^{a}	6.7±2.2^{a}	6.3±1.1^{b}	7.3±1.6^{a}
	0.5	7.5±.1.5^{a}	7.1±1.1^{a}	7.2±1.2^{a}	6.6±1.2^{b}	7.5±.1.5^{a}
	1	7.1±1.5^{a}	7.2±1.1^{a}	6.2±2.2^{a}	6.1±1.2^{b}	7.1±1.1^{a}
	1.5	6.8±2.1^{a}	6.8±1.6^{a}	6.2±1.2^{a}	6.2±1.5^{b}	6.8±1.1^{a}
	2	7.1±1.8^{a}	6.5±2.2^{a}	6.9±2.1^{a}	8.2±0.2^{a}	7.1±1.2^{a}
	2.5	7. 5±1.5^{a}	4.3±0.8^{b}	7.1±1.3^{a}	7.1±0.9^{ab}	5. 1±0.5^{b}
8	0	5.2±1.1^{b}	7.1±1.7^{a}	6.2±2.1^{a}	5.6±1.2^{b}	5.4±0.4^{b}
	0.5	6.1±0.8^{ab}	6.9±1.1^{a}	7.1±1.1^{a}	5.2±1.4^{b}	5.1±2.8^{ab}
	1	7.2±1.2^{a}	6.8±1.2^{a}	7.4±1.2^{a}	5.1±1.8^{b}	6.2±1.1^{a}
	1.5	7.7±1.3^{a}	7.0±1.4^{a}	6.2±1.8^{a}	5.7±1.1^{b}	6.7±1.3^{a}
	2	7.1±1.9^{a}	6.3±1.2^{a}	7.2±1.3^{a}	8.1±0.2^{a}	7.1±1.1^{a}
	2.5	7.8±1.2^{a}	4.4±0.5^{b}	7.1±1.3^{a}	7.6±0.4^{a}	5.8±1.2^{ab}
13	0	3.1±0.9^{b}	7.3±1.1^{a}	5.6±2.2^{a}	4.1±0.8^{c}	3.1±0.9^{b}
	0.5	4.5±1.2^{b}	7.1±1.3^{a}	6.4±1.6^{a}	4.5±1.1^{c}	4.5±1.2^{b}
	1	5.4±1.3^{ab}	7.1±2.1^{a}	7.2±1.8^{a}	5.1±1.1^{bc}	5.4±0.3^{ab}
	1.5	6.6±1.2^{a}	7.1±1.3^{a}	6.4±1.9^{a}	5.5±0.2^{b}	6.6±1.2^{a}
	2	7.2±1.8^{a}	6.5±1.2^{a}	6.3±1.9^{a}	7.6±1.6^{a}	7.2±1.8^{a}
	2.5	7.3±1.8^{a}	4.2±1.6^{b}	7.1±1.2^{a}	7.3±1.5^{a}	5.3±1.8^{ab}
16	0	1.1±0.9^{b}	5.1±1.1^{a}	NA	2.3±0.3^{d}	1.1±0.9^{c}
	0.5	2.2±0.8^{b}	5.8±1.1^{a}	NA	3.1±1.1^{cd}	2.2±0.6^{c}
	1	3.1±1.1^{b}	6.2±1.2^{a}	5.3±3.4^{a}	4.2±1.2^{c}	4.1±1.1^{b}
	1.5	5.2±1.3^{ab}	6.2±1.2^{a}	5.9±2.1^{a}	5.2±0.4^{b}	6.2±0.8^{b}
	2	7.8±1.2^{a}	6.4±1.3^{a}	6.4±2.6^{a}	6.5±1.9^{a}	7.8±0.2^{a}
	2.5	7.1±1.8^{a}	4.4±0.4^{b}	7.4±1.8^{a}	6.3±1.3^{a}	5.1±1.8^{b}
21	0	1.2±0.5^{c}	4.1±1.1^{b}	NA	2.1±1.1^{c}	1.2±0.5^{c}
	0.5	1.1±0.9^{c}	4.2±1.1^{b}	NA	2.4±2.3^{c}	1.1±0.9^{c}
	1	3.2±0.9^{c}	5.3±1.2^{ab}	NA	5.5±1.8^{bc}	3.2±0.9^{c}
	1.5	3.8±1.3^{c}	5.3±1.2^{ab}	NA	5.1±0.7^{b}	3.8±1.3^{bc}
	2	7.1±1.1^{a}	6.9±1.3^{a}	6.8±2.2^{a}	6.1±0.4^{a}	7.1±1.1^{a}
	2.5	7.2±0.5^{a}	4.4±0.3^{b}	6.9 ±2.2^{a}	6.9±0.4^{a}	5.2±0.5^{b}

Data are expressed as mean ± standard deviation (n=9). Mean values in the same row bearing different superscripts are significantly different ($p < 0.05$).



Fig

Fig 26 Effect of radiation treatment (2 kGy) and storage on L values of RTC cabbage at A. 10 °C and B. 4 °C. Samples stored at 10 °C and 4 °C were studied for 16 days and 21 days respectively.

3.4.2.3 Texture Analysis

Loss in firmness of vegetable often affects consumer acceptability. Fig 27C & 27D provides the puncture strength of both the control and irradiated (2 kGy) samples stored for different days at two different temperatures (10 °C and 4 °C). A slight decrease in puncture strength immediately after irradiation with no further decrease in firmness thereafter until the end of the storage period was noted. This may be due to radiation induced depolymerization of pectin and other cell wall components such as cellulose and hemicelluloses leading to softening [141]. However, this did not affect the overall acceptability of the sample. The irradiated sample (2 kGy) retained its texture throughout the storage period. This may be due to lower transpiration rate in irradiated than in the control sample. In contrast, in the control sample, the firmness remained unaffected up to a storage period of 13 days beyond which this parameter decreased significantly. The data obtained are in agreement with the scores on texture provided by the sensory panel. The decrease in puncture strength at later period of storage in control sample could be due to its decay. A loss of firmness when stored in air after 12 days of storage resulting from a possible increased proliferation of pectolytic pseudomonas has also been reported by Amanatidou et al [145]. Fan et al [146] on the other hand reported that irradiation had no effect on the firmness of minimally processed iceberg lettuce. No significant effect of radiation processing on firmness was also noted by Khattak et al [143] in minimally processed cabbage and cucumber when stored at refrigeration temperatures (5^{o}C). In the present study, from the view point of texture, 2kGy sample was thus found to be well acceptable.

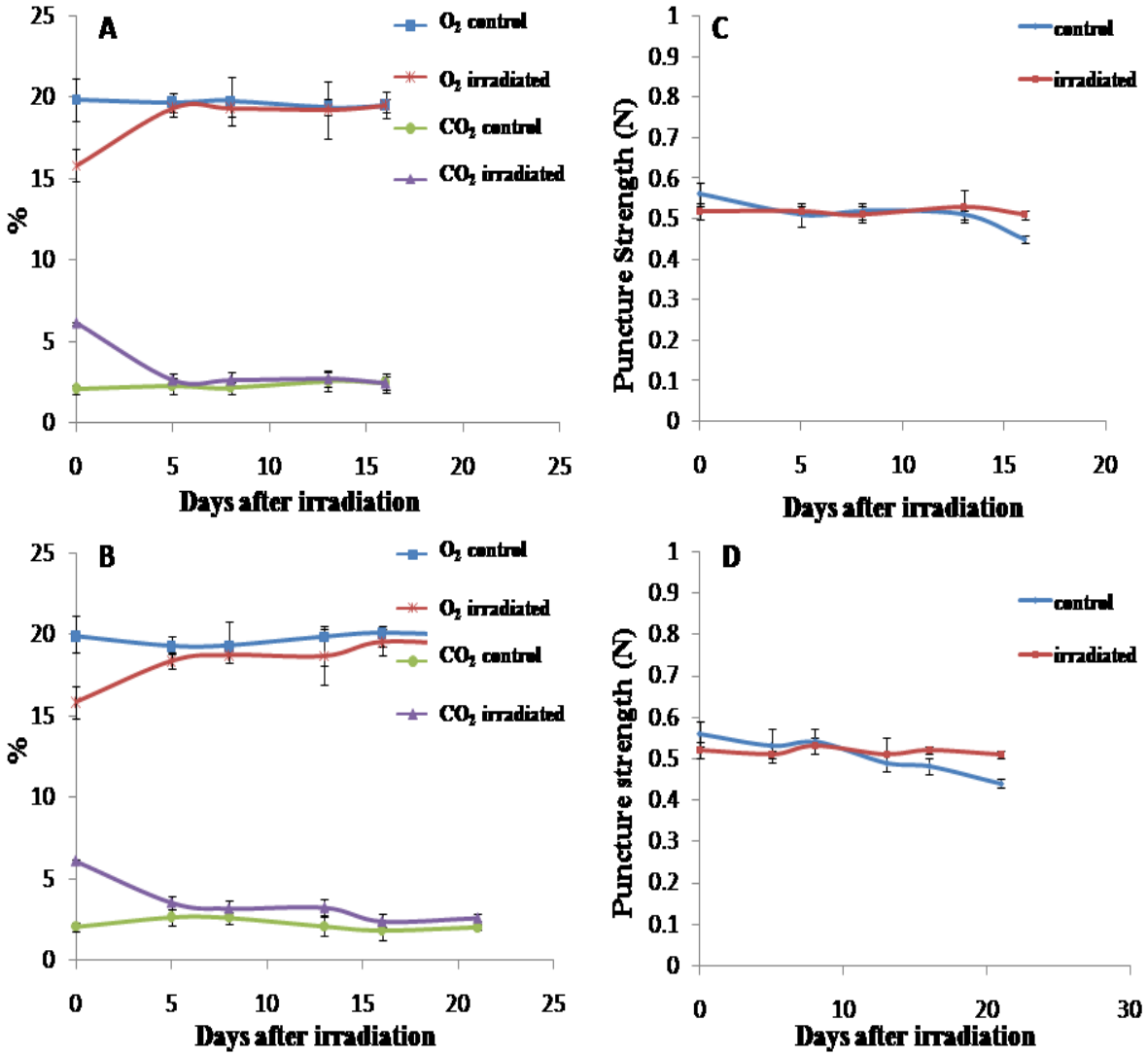


Fig. 27. Variation of O_2 & CO_2 content in headspace of packets during storage at **A**. 10 ° C and **B**. 4 °C; Puncture strength (N) of RTC cabbage during storage at **C**. 10 ° C and **D**. 4 ° C. Values are expressed as mean ± SD (n=9). Samples stored at 10 °C and 4 °C were studied for 16 days and 21 days respectively.

3.4.3 Headspace gas composition

Headspace gas composition forms an important aspect in the storage of minimally processed vegetables. A very high O_2 content often leads to increased unfavorable reactions like browning while a lower O_2 level leads to growth of anaerobic bacteria. Hence, maintenance of optimum balance between O_2 and CO_2 level is very crucial. Significant ($p \leq 0.05$) effect of irradiation and storage time on the head space composition of both the gases was observed during storage (Fig 27A & 27B). Similar trend was seen for both gases stored at 4 °C and 10 °C. O_2 and CO_2 concentration (18% and 6% respectively) remained constant in control samples throughout the storage period. A significant decrease in O_2 content and an increase in CO_2 content were noted immediately after irradiation. O_2 level less than 2% is known to favor growth of anaerobic bacteria like *Clostridium botulinum* and microaerophilic bacteria like *Listeria spp.* and lactic acid bacteria [147]. In the present study, the lowest O_2 content observed was 15.8 % immediately after irradiation. This may be because of rapid increase in respiration rate due to rise in cellular activity after irradiation. Aerobic condition was thus maintained throughout the storage period. On further storage, the O_2 content gradually increased and CO_2 content decreased reaching the level in the control on day 5 and then remained constant throughout the storage period. A similar observation in irradiated mushroom resulting from reduction in metabolic activity during storage has been demonstrated. Elevated CO_2 levels have been shown to extend lag phase and thus slow the propagation of bacteria [148]. The enhanced CO_2 levels during the initial storage period in the irradiated samples may thus aid in slowing down microbial growth and thus improving shelf life compared to the control samples. The maintenance of constant O_2 and CO_2 level beyond a storage period of 5 days may be due to attainment of equilibrium condition between package headspace and atmosphere with time. A similar observation was also noted earlier in

irradiated minimally processed ash gourd [149].The headspace gas composition was therefore found to be suitable for storage of RTC cabbage.

Thus 2 kGy dose was thus found to be optimum for shelf life extension of cabbage both at 10^0C and 4^0C. The nutritional quality of the vegetable with respect to radical scavenging activity, total phenolics, total flavonoid and vitamin C content was further evaluated at this dose.

3.4.4 Analysis of nutritional quality

Wholesomness of irradiated food has been extensively investigated and reported [52]. Studies on nutritional adequacy of irradiated foods have also shown that irradiation at any dose level that is of practical interest i.e. about 50 kGy do not impare the nutritional properties of major food components namely carbohydrate, proteins and fat. However, changes in vitamins such as ascorbic acid have been reported. Besides, compounds that contribute to antioxidant properties such as phenolics including flavonoids have also been known to undergo variation in their content during irradiation. Hence changes in vitamin C and phenolic compounds were investigated.

3.4.4.1 Total phenolic and flavonoid content

Phenolics and flavonoids form important nutritional constituents of vegetables. Preserving these compounds in the vegetable is therefore essential for maintaining their quality. Radiation induced increase in phenolic content has been previously demonstrated in vegetables like carrot and kale juice [51], fresh-cut iceberg lettuce [152] and ash gourd [149]. Villavicencio et al. on the other hand reported a radiation induced reduction in phenolic content at 10 kGy in Macacar bean [153]. Reduction in phenolic content in Chinese cabbage beyond a dose of 1 kGy has also been reported [138]. In the present study, the phenolic

content was found to be comparable to that reported earlier for cabbage (70-75 GAE mg/g FW) [154]. Beyond 10 days of storage a decrease in total phenolic content was noted in both the control and irradiated samples (Fig. 28E) (58-60 GAE mg/g FW). No significant effect on the phenolic content was, however, noted as a result of irradiation and storage temperatures. The flavonoid content was also not found to be affected by irradiation and storage (Fig 28F) (60 to 65 QE μg/g). The content of flavonoid estimated in the present study is comparable with the values reported in literature [154].

3.5.4.2 Vitamin C analysis

Vitamin C is the most sensitive vitamin being degraded quickly on exposure to heat, light and oxygen. It acts as an antioxidant in the body by protecting against oxidative stress and is also a cofactor in several key enzymatic reactions. Variation in the vitamin C content among different cabbage cultivars ranging from 5.7 to 23.5 mg/100 g has been reported by Singh et al[155]. The amount estimated in present study is in agreement with the previous reports available (17-18 mg/100 g FW). The content of vitamin C was found to be unaffected by radiation processing and storage (Fig 28 G). .A similar observation was earlier reported in irradiated ash gourd [149]. Thus the nutritional quality of RTC cabbage with respect to antioxidant activity, phenolic and flavonoid content and vitamin C status was maintained upon irradiation.

3.5.4.3 Anioxidant activity

Fresh fruits and vegetables are known to possess significant antioxidant activity. However, processing and storage may deteriorate the inherent antioxidant activity of the product. Hence assessment of effect of processing and storage on antioxidant activity forms an important parameter for development of RTC vegetables from a nutritional view point. No single antioxidant assay reflects the total antioxidant capacity of the compounds present in the

product. So a number of antioxidant assays involving different principles were studied in RTC cabbage.

3.5.4.3.1 DPPH activity

The DPPH• test is usually used to provide basic information on the ability of extracts to scavenge free radicals. Gamma irradiation was found to enhance DPPH• radical scavenging activity of RTC cabbage (Fig 28A) from 150 to 170 GAE μg/g. It is known that irradiation generates free radicals that may act as stress signals and trigger stress responses in vegetables, resulting in increased antioxidant synthesis [146]. A similar phenomenon in present case could account for the increased antioxidant activities observed in the irradiated vegetable. However, beyond ten days, the activity declined in both control and irradiated samples which might be due to degradation of antioxidants as a result of oxidation on prolonged storage. Similar trend in antioxidant activity was also noted at both the storage temperatures studied.

3.5.4.3.2 Reducing power analysis

The reducing power of a compound may serve as a significant indicator of its potential antioxidant activity. Hence, effect of irradiation and storage on the Fe^{3+}-reducing powers of methanolic extract was investigated. No difference was seen in reducing power values for control and irradiated sample with storage time and temperature (Fig 28B). Cabbage is known to possess significant reducing power. In the present case ferric reducing power of the cabbage was found to lie between 50 to 55 AEAC μg/g. Effect of irradiation and storage has not been reported earlier for cabbage samples. The present study shows that gamma irradiation is an effective tool for maintenance of this property of cabbage.

3.5.4.3.3 Hydroxyl radical scavenging activity

Reactive oxygen metabolites, including free radicals such as nitric oxide (NO) and hydroxyl (OH) radicals are toxic and play an important role in tissue injury. Hydroxyl radical is the

most reactive radical known. It can attack and damage large number of molecules found in living cells. In the present study an increase in hydroxyl radical scavenging activity was found with irradiation (Fig 28C) from 120 to 140 GAE μg/g. Allyl isothiocyanate, the major volatile compound of cabbage is known to possess significant hydroxyl radical scavenging activity[150]. An increase in allyl isothiocyanate due to gamma irradiation demonstrated by us in the present study may account for the enhancement in hydroxyl radical scavenging activity in the irradiated sample.

3.5.4.3.4 Nitric oxide radical scavenging activity

Reactive nitrogen species (RNS) like nitric oxide (NO) free radical is associated with several types of cellular damage including lipid peroxidation, protein oxidation and nitration, enzyme inactivation, and DNA damage. No significant effect was noted as a result of irradiation, storage or variation in temperature on nitric oxide scavenging activity (Fig 28 D). NO radical scavenging activity of the samples was found o lie between 60 to 65 QE μg/g. Previous reports on cabbage have indicated similar results.

Thus radiation processing at a dose of 2 kGy extended shelf life of RTC cabbage 8 days at 10 ºC thereby improving the feasibility of its storage of such products at higher temperature. The product was found to be microbiologically safe and had acceptable sensory and nutritional quality. Radiation processing can therefore aid in enhancing shelf life of minimally processed vegetables when stored at higher temperatures in supermarkets thereby saving on both energy and cost besides increasing its marketability.

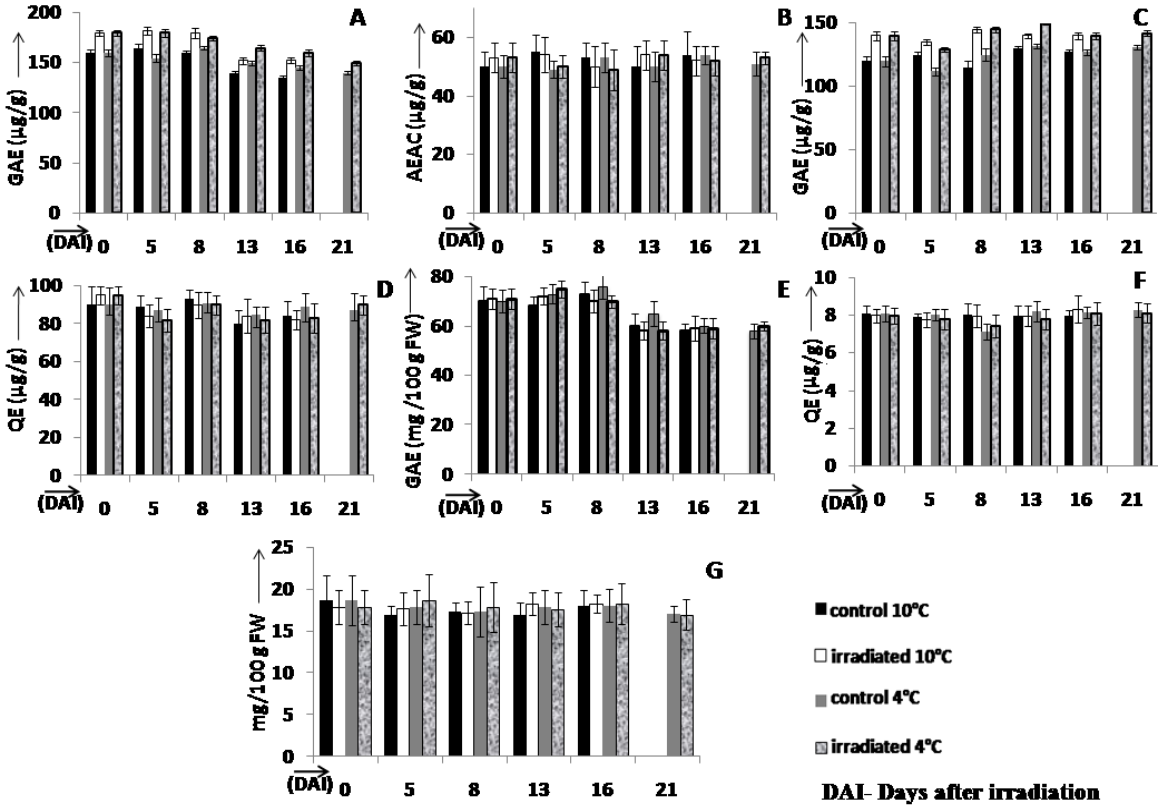


Fig 28 Effect of radiation processing (2 kGy) and storage on nutritive quality of RTC cabbage. **A.**DPPH radical scavenging activity; **B**. Ferric reducing property; **C**. Hydroxyl radical scavenging activity; **D**. Nitric oxide radical scavenging activity. **E**. Total phenolic content; **F**. Total flavonoid content; **G**. Vitamin C content. Values are expressed as mean ± SD (n=9). Samples stored at 10 °C and 4 °C were studied for 16 days and 21 days respectively.

3.6 Gamma irradiation induced browning inhibition

Studies so far have demonstrated the feasibility of using gamma irradiation for extending shelf life of minimally processed cabbage. Fig 29 provides a representative photograph of the product so developed. It is evident from the figure that the irradiated minimally processed shredded cabbage has a superior visual appeal compared to the control samples as a consequence of browning inhibition at the cut edges. Among the physiological factors limiting post harvest storage of fresh plant produce, enzymatic browning plays a major role in reducing sensory quality and nutritional value of these products. Gamma irradiation induced browning inhibition in cut vegetables has been previously reported by some workers but the mechanism of its inhibition in cut vegetables has not been documented so far.

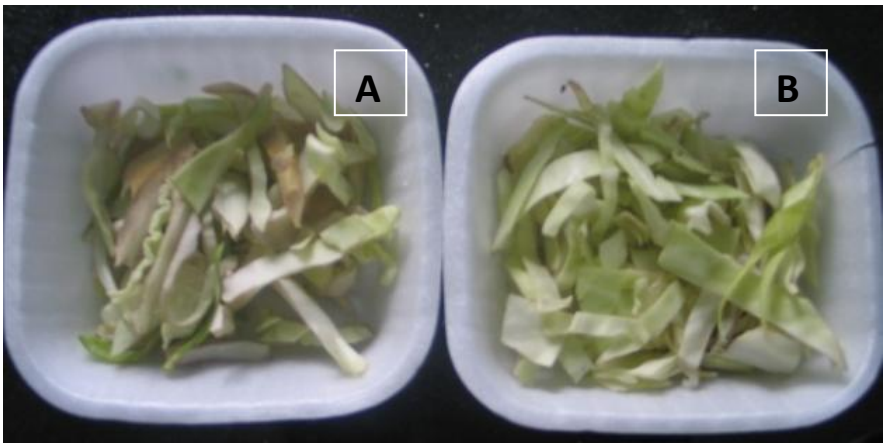


Fig 29 Minimally processed cabbage samples stored at 10 °C after 16 days of storage

Browning in cut fruits and vegetables mainly involves metabolism of phenolic compounds into their oxidized products [24,25]. In intact plants, phenolic compounds in cell vacuoles are spatially apart from the oxidizing enzymes present in the cytoplasm. Once tissues are damaged by cutting, grinding or pulping, the rapid mixing of the enzymes and phenolic compounds as well as the easy oxygen diffusion to the inner tissues results in a browning reaction. In response to tissue injury phenylalanine ammonia lyase (PAL) produces phenols which are then oxidized by polyphenol oxidase (PPO) and peroxidase (POD) to o-quinones that further polymerize to brown pigments.

Gamma-radiation induced inhibition of browning in minimally processed shredded cabbage stored (10°C) up to 8 days was investigated as the control samples were badly spoiled beyond 8 days. Fresh cabbage samples were processed as detailed in section 2.8. Various parameters responsible for enzymatic browning such as enzyme activities (PPO, PAL and POD), phenolic composition and o-quinones were monitored before and after radiation treatment. No significant difference in any of the measured parameters was observed during cold storage (up to 8 days) in whole heads of cabbage. Therefore, any change in the fresh-cut vegetable observed during storage could be inferred as an effect of shredding.

3.6.1. Enzymatic browning in non-irradiated shredded cabbage

3.6.1.1 Evaluation of browning

The effect of irradiation and storage on browning at the cut edges of cabbage is shown in Figure 30. The sensory score (Figure 30 B) and the L value (Figure 30 A) measured by colorimeter demonstrated similar results. Significant browning at the cut edges was seen in control samples beyond 4 days of storage which further increased by the end of 1 week. (Fig 30 A & B) Cut edge browning on storage has been reported in a number of vegetables thus reducing the shelf life of the products [144].

3.6.1.2 Evaluation of enzyme activities

Alteration in phenolic metabolism is generally known to affect browning in cut vegetables. PAL is the first enzyme in the phenylpropanoid pathway involved in synthesis of phenolic compounds. In the present study a low PAL activity was observed in the freshly cut cabbage strips. With storage, the activity was found to increase, reaching maxima on day 2 and then remaining constant up to day 4, after which a slight decrease was noted on further storage (Fig31A). Several studies on cut lettuce have shown a wound induced enhancement in PAL activity. Degl Innoceti [103] for instance noted a significant increase in PAL activity within 5 hours, whereas Hisaminato et al [156] found maximum increase after 3 days of storage. Murata et al [157] also found a significant increase in the activity of this enzyme after 3 days of storage that further increased on storage up to day 6. Thus, the effect of wounding on PAL activity was found to vary with the variety of lettuce. Stress induced enhancement in PAL activity has been extensively reported in different plant tissues. Various stresses, such as nutrient deficiencies, viral, fungi, and insect attack are known to increase either PAL synthesis or activity in different plants [24]. In the present case shredding of cabbage induced a stress which resulted in an increase in PAL activity. Wound induced enhancement in PAL activity has also been previously reported in minimally processed potatoes [158].

PPO is a downstream enzyme in the phenylpropanoid pathway acting on phenols to form o-quinone. PPO activity remained nearly constant during storage in the present study (Table 23). This indicated that PPO activity was high enough in shredded cabbage to cause browning. Other authors have also reported no significant changes in PPO activity in iceberg lettuce leaf cuts during cold storage[103, 156].

POD is another enzyme almost ubiquitously present in plant, that in the presence of hydrogen peroxide convert a number of phenolics to form o-quinione. However, its role in enzymatic

browning remains questionable mainly because of the low H_2O_2 content in vegetable tissues[24]. Free radicals including H_2O_2 are generated due to water radiolysis on irradiation. Thus analysis of POD activity is of significance in the present study. POD activity was assayed in the presence of natural hydrogen donors (caffeic and chlorogenic acid). POD activities did not vary substantially during storage for both the substrates (Table 23) thus ruling out its role in browning in shredded cabbage.

3.6.1.3 Transcriptional analysis of PAL gene expression

Shredding and storage in cabbage samples resulted in a change in PAL activity in cabbage samples while no change was noted in the activities of other enzymes. Transcriptional analysis of PAL gene at different storage points was therefore studied. Gene expression was analyzed at 0, 24 and 48 hrs of storage. The expression level of these transcripts at various storage periods was recorded. A comparison of the expression levels of the control sample at 0 hr (Fig. 32) with those at 24 and 48 hrs showed a gradual increase in PAL gene expression with storage. An up-regulation of 1.2 fold and 7.7 fold was seen after 24 hours and 48 hours respectively thus justifying the increase in PAL activity during storage. Similar results were seen in cut lettuce where a 3.4 fold increase was seen in PAL mRNA within 24 hours of wounding which resulted in increase in PAL activity.

3.6.1.4 Analysis of phenolic content

PAL catalyzes the biosynthesis of phenolic compounds. Since these compounds play a major role in browning, their nature and content in the vegetable was further analyzed. Table 24 lists the major phenolic compounds identified in shredded cabbage. Gallic acid was found to be the major phenolic acid followed by γ-resorcylic acid and chlorogenic acid. Ferulic acid, sinapic acid and ellagic acid were detected in minor amounts. Amongst these, chlorogenic acid and sinapic acid have been identified in different cabbage [158] while ferulic acid has

been demonstrated to exist in cabbage as quercetin and kaempferol derivative [159]. Gallic acid, γ-resorcylic acid and ellagic acid have, however, not been previously reported in cabbage.

The total phenolic content was found to be comparable to that reported earlier for cabbage (Figure 33). Interestingly, no change in the total phenolic content was observed during the entire storage period of 8 days. Similar results were seen in cut chicory where a significant increase in PAL activity was noted due to wounding with no change in phenolic content [160]. Degl'Innocenti [161]also found similar results in lettuce and escarole wherein, an increase in the enzyme activity was noted with storage with no change in the content of phenolic compounds. Several studies have shown that accumulation of phenolic compounds in plant cell is not a mere function of the rate of phenolic synthesis but varies strongly in relation to its physiological state and is a result of equilibrium between biosynthesis and further metabolism including turnover and catabolism [162]. In the present study, we did not find any correlation between phenolic content and browning. Thus equilibrium between phenolic biosynthesis and its further metabolism could possibly explain the absence of any change in the total phenolic content. Our results are in accordance with the reports of Hisaminato et al [156] and Vitti et al [163] for cut lettuce and potato respectively where no correlation could be established between phenolic content and browning.

3.6.1.5 Analysis of o-quinone content

Phenols are known to be converted to o-quinones by PPO which in due course either polymerize and/or combine together with amino compounds to form high molecular weight brown pigments resulting in browning [164]. Therefore, o-quinone content of the cabbage samples was evaluated further. In the control samples an increase in its content was seen with storage reaching its maxima on day 4 (Figure 34). The quinone content showed good

correlation (R^2=0.99) with PAL activity (Figure 34) as well as with browning, thus further establishing a direct relationship between increase in PAL activity and browning.

3.6.2 Effect of γ-irradiation on cut edge browning in shredded cabbage

3.6.2.1 Evaluation of browning

Gamma irradiation was found to have an inhibitory effect on browning. With an increase in irradiation dose the extent of browning was found to decrease. In the 0.5 kGy treated samples, browning of cut edges was observed beyond 6 days of storage; while in the samples exposed to a dose of 1 kGy browning could be observed only at the end of storage period (8 days). Samples exposed to a dose of 2 kGy appeared fresh and had no visual browning throughout the storage period. Similar observation has been made by Ke and Saltveit [18] in various fresh-cut vegetables wherein gamma radiation dose dependent inhibitory effect on browning was noted. Similar observation has been made by Tripathi et al [149], where a dose of 2 kGy was found to be effective in inhibiting cut edge browning in ash gourd cubes. On the other hand, irradiation induced browning has been reported in potato tubers, mushrooms, tropical fruits and in cut witloof chicory wherein a dose of 3 kGy was found to induce browning during storage [160,165]. Tanaka et al [160] described the browning process to be non enzymatic arising due to generation of free radicals on irradiation. Hanotel et al [165], on the other hand, found an increase in PAL activity during gamma irradiation to be responsible for the enhanced browning observed. In view of the contradictory observations, a detailed study on the browning process is envisaged for a better understanding of the browning inhibition during radiation processing as currently observed.

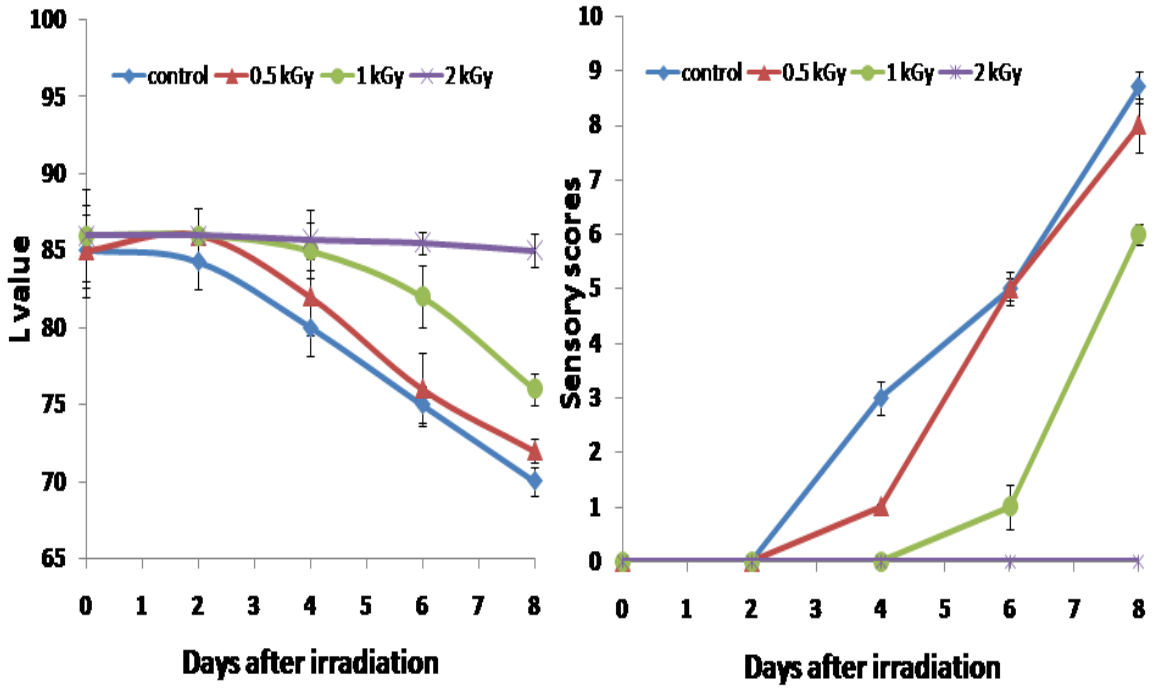


Figure 30 A) Effect of radiation treatment on L values B) Effect of radiation treatment on sensory score for browning.

3.6.2.2 Evaluation of enzyme activities.

Effect of gamma irradiation and storage on PAL activity in shredded cabbage is shown in Fig. 31A. A slight increase was observed in PAL activity at 0.5 kGy immediately after irradiation which increased gradually by day 4 and then remained constant. Induction of PAL activity at low dose of γ-irradiation has been previously reported by Pendharkar et al [166]. At 1kGy, PAL activity was not significantly affected immediately after irradiation, however increase in activity was observed from day 6 onwards. A small but significant decrease was noted in the PAL activity immediately after irradiation at 2 kGy that remained unchanged with further storage. Since, significant browning was seen in control samples from day 4 onwards the enzyme activity in samples treated with different doses were compared on this day (Fig. 31B). A dose dependent decrease was seen in PAL activity thus confirming the role of γ-irradiation

in inhibiting PAL activity. No effect of γ-irradiation on PPO and POD activities were observed (Table 23). Benoit et al [167] also found a decrease in PAL activity in mushrooms resulting in retention of whiteness of the sample. Our results are in contrast to most of the earlier reports on cut products such as witloof chicory, potato tubers and litchi pericarp wherein an increase in PAL activity due to γ-irradiation has been reported [165,166].

A positive correlation (R^2=0.97) between browning and PAL activity (Fig 31C) was noted. No such correlation was, however, found with PPO and POD activity. Thus, suppression of PAL activity by gamma irradiation was postulated to be the major reason behind browning inhibition. A positive correlation between PAL and browning and no significant correlation with the other two enzymes has been previously reported in cut potatoes [25]. Hisaminato et al [156] observed a clear relationship between browning and PAL activity in cut lettuce during storage. They further reported that browning can be prevented by inhibiting PAL activity. Murata et al [157] found an inhibition in browning in cold stored cut lettuce previously subjected to heat shock treatment (50°C, 90 s). They found that the heat shock significantly repressed the induction of PAL activity thus preventing browning. Many authors have claimed that PAL activity can be used as an index to judge the extent of damage in fresh-cut produce, in particular color and texture, during processing and thus estimate their shelf life and quality [27,28].

3.6.2.3. Transcriptional analysis of PAL gene expression

Since a dose of 2 kGy was found to be effective in inhibiting browning in shredded cabbage throughout storage, transcriptional analysis of PAL gene was performed at this dose. Gene expression in control and irradiated samples was analyzed at 0, 24 and 48 hrs after irradiation. The expression level of these transcripts at 24 and 48 hrs was compared with their expression in control sample at 0 hr (Fig. 32). Irradiated sample showed a down-regulation by 1.4 fold

immediately after irradiation that remained constant on further storage up to 48 hours. This could very well explain the lower but constant PAL activity in irradiated sample. A similar effect on PAL activity has also been reported earlier during heat shock treatment in several produce [168,169]. Heat shock treatment in lettuce resulted in inhibition of PAL activity by hindering the accumulation of PAL proteins either by preventing the translation or accelerating the turnover of PAL proteins [168]. Heat shock induced thermal degradation of PAL mRNA has also been reported in yeast [168] and cereal seeds [169]. Thus mechanism of suppression in activity can vary with the nature of the produce.

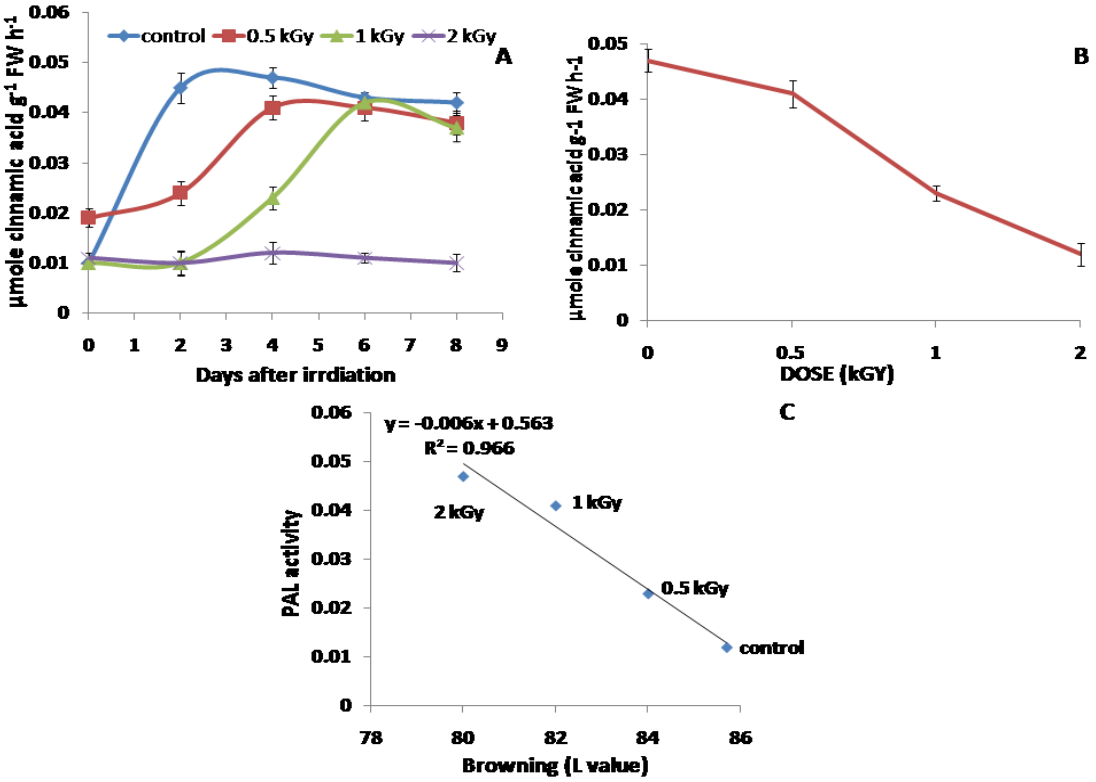


Fig 31 A) Effect of irradiation and storage (8 days) on PAL activity. B) PAL activities at different doses on day 4. C) Plot depicting the relation between PAL activity and browning in shredded cabbage.

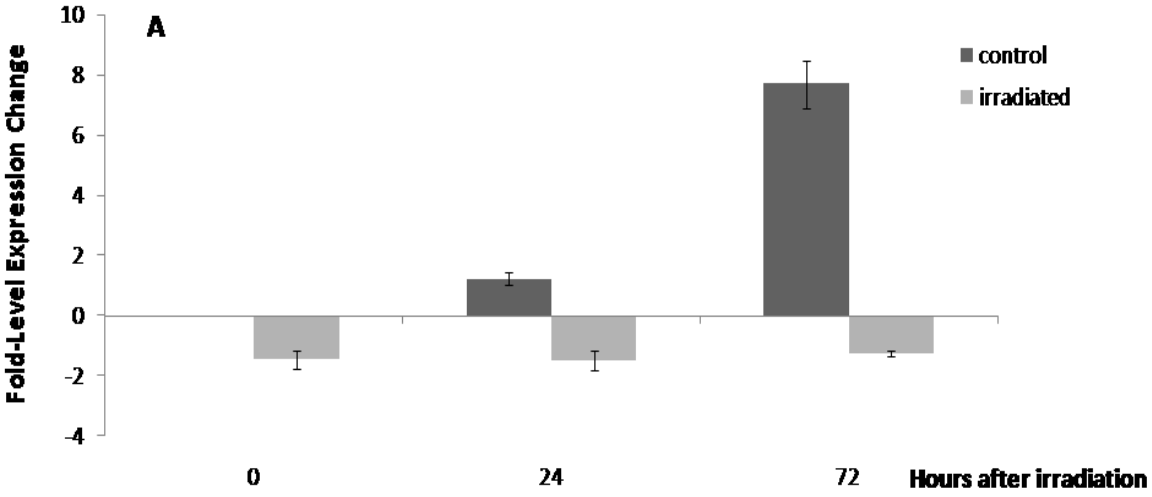


Fig 32 Fold change in the expression of PAL gene. The x-axis represents the expression level of PAL gene in control at 0 hr. All values are means of triplicates ±SD.

Table 23 Effect of irradiation and storage on PPO and POD activity of cabbage

Data are expressed as mean ± standard deviation (n = 9). Mean values in the same column bearing

DAY	Control			0.5 kGy			1 kGy			2 kGy		
	PPO	POD1	POD2	PPO	POD1	POD2	PPO	POD1	POD2	PPO	POD1	POD2
0	10.1±2.1^{a}	6.1±1.1^{a}	5.4±2.2^{a}	11.1±2.2^{a}	6.9±1.9^{a}	4.9±1.8^{a}	12.1±4.1^{a}	7.1±2.4^{a}	5.1±1.1^{a}	10.1±3.9^{a}	6.9±1.9^{a}	4.6±1.1^{a}
2	11.1±4.7^{a}	7.2±2.2^{a}	6.1±1.8^{a}	10.7±3.1^{a}	6.7±1.7^{a}	5.3±1.2^{a}	12.3±1.1^{a}	6.8±1.8^{a}	5.2±1.2^{a}	11.1±2.1^{a}	5.9±2.1^{a}	5.1±2.1^{a}
4	12.4±3.1^{a}	6.3±1.9^{a}	5.1±1.6^{a}	12.1±4.2^{a}	5.8±2.1^{a}	5.9±2.2^{a}	10.5±2.8^{a}	5.9±1.9^{a}	4.9±2.1^{a}	12.1±3.3^{a}	6.1±2.3^{a}	4.9±1.3^{a}
6	11.1±2.1^{a}	5.8±2.1^{a}	4.1±1.4^{a}	10.7±2.1^{a}	6.1±2.2^{a}	6.1±2.3^{a}	11.1±4.7^{a}	7.1±2.1^{a}	5.2±0.7^{a}	10.5±3.8^{a}	6.7±1.9^{a}	6.1±1.5^{a}
8	10.2±3.9^{a}	6.4±2.3^{a}	4.8±2.1^{a}	10.9±3.1^{a}	6.4±1.2^{a}	4.9±1.7^{a}	10.9±2.3^{a}	6.3±2.2^{a}	4.9±1.1^{a}	11.1±4.1^{a}	5.8±2.1^{a}	4.9±1.3^{a}

same superscript shows no significant difference ($p \leq 0.05$). PPO activity is represented in U g^{-1}FW, POD activity is represented in Δ A min-^{1} g^{-1} FW; POD1 = caeffic acid peroxidase activity and POD2 = chlorogenic acid peroxidase activity.

3.6.2.4 Analysis of phenolic content

Total and individual phenolic content remained unaffected during irradiation and subsequent storage (Fig 33). This could be explained by the constant PAL activity in the irradiated samples. Pinaka et al [56] have previously shown that the content of phenolic compounds remain unaffected in irradiated broccoli and carrots. Absence of any change in the phenolic content in the irradiated samples as observed here may have a positive effect on the nutritional quality of shredded cabbage.

3.5.2.5 Analysis of o-quinone content

Similar to PAL activity o-quinone content also decreased with increase in irradiation dose (Fig 34A). In samples exposed to a dose of 0.5 kGy *o*-quinone content was found to increase from day 4 onwards while in 1 kGy sample this increase was noted on the 8th day. No change ($p \leq 0.05$) in soluble *o*-quinone content was found throughout the storage period in samples given a dose of 2 kGy. The quinone content showed good correlation ($R^2=0.99$) with PAL activity (Fig. 34B) as well as with browning, thus further establishing a direct relationship between increase in PAL activity and browning. In irradiated sample (2 kGy) as with PAL activity, no change in o-quinone content was seen throughout the storage period. Similar results were seen in cut roman lettuce exposed to heat shock [170].

Thus, gamma irradiation caused a down regulation in PAL gene expression consequently lowering PAL activity and thus inhibiting cut edge browning in shredded cabbage.

Table 24 Effect of gamma irradiation (2 kGy) on phenolic acids content (mg/kg) in shredded cabbage

PHENOLIC ACID	DAY 0		DAY 2		DAY 4		DAY 6		DAY 8	
	c	I	C	I	C	I	C	I	C	I
Gallic acid	28.1± 4.5[a]	29.3± 5.8[a]	29.1± 4.5[a]	28.3± 3.5[a]	29.6± 4.5[a]	28.1± 6.6[a]	28.1± 4.5[a]	27.3± 4.9[a]	29.4± 2.3[a]	29.5± 3.1[a]
γ-Resorcylic acid	6.3± 1.1[b]	6.1± 2.1[b]	5.9± 1.2[b]	6.2± 1.4[b]	5.9± 1.1[b]	5.7± 1.5[b]	6.2± 1.5[b]	5.7± 0.9[b]	5.8± 1.6[b]	6.2± 1.2[b]
Chlorogenic acid	4.2± 1.2[c]	3.8± 0.8[c]	3.8±1.1[c]	4.1± 1.1[c]	4± 1.3[c]	3.7± 1.7[c]	4.2± 1.2[c]	3.8± 1.1[c]	4.1± 0.9[c]	4.4± 1.2[c]
Ferulic acid	1.5± 0.4[d]	1.1± 0.8[d]	1.8±0.8[d]	1.3± 0.5[d]	1.3± 0.6[d]	1.7± 0.8[d]	1.2± 0.4[d]	1.3± 0.9[d]	1.2± 0.9[d]	1.5± 0.7[d]
Sinapic acid	1.2± 0.2[e]	1.4± 0.4[e]	1.1±0.3[e]	1.2± 0.6[e]	1.6± 0.5[e]	1.4± 0.5[e]	1.2± 0.2[e]	1.1± 0.5[e]	1.5± 0.2[e]	1.3± 0.2[e]
Ellagic acid	1.5± 0.4[f]	1.3± 0.4[f]	1.2± 0.4[f]	1.1± 0.5[f]	1.4± 0.6[f]	1.6± 0.4[f]	1.1± 0.2[f]	1.1± 0.3[f]	1.2± 0.6[f]	1.1± 0.4[f]

Data are expressed as mean ± standard deviation (n = 9). Mean values in the same column bearing same superscript shows no significant difference ($p \leq 0.05$).
c=control I=irradiated

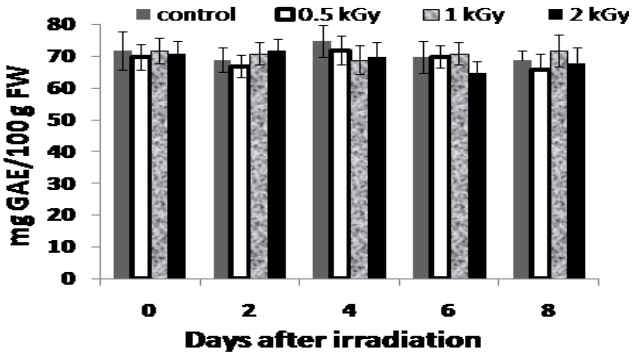


Fig 33 Effect of irradiation and storage on total phenolic content

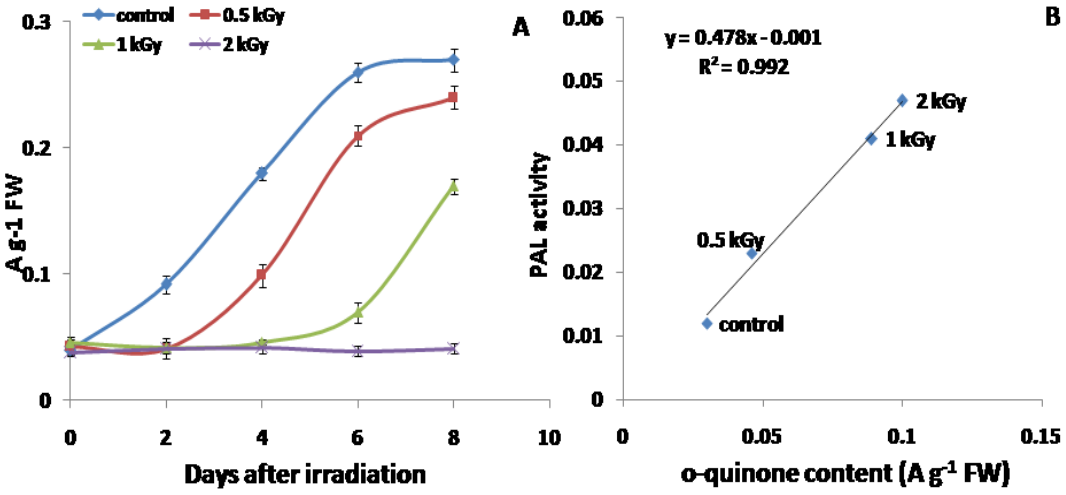


Fig 34 A. Effect of irradiation and storage on soluble o-quinone content in shredded cabbage. B. Plot depicting the relation between PAL activity and o-quinone content in shredded cabbage**.** PAL activity and o-quinone content were evaluated on 4th day of storage. Values are expressed as mean ± SD (n=9).

3.6.3 Effect of γ-irradiation on non-enzymatic browning in shredded cabbage

Non-enzymatic browning in vegetables, although of less significance, can also occur during storage. ASA, present in appreciable amounts in vegetables, is known to get converted non-enzymatically to DHA on storage that can get degraded to brown pigments. Degl'innocenti et al [103] have reported a liner correlation between conversion of ASA to DHA and the occurrence of browning in fresh-cut lettuce leaves. Therefore the content of ASA and DHA in control and irradiated samples during storage was investigated. The content of ASA has been reported to vary among different cabbage cultivars from 5.7 to 23.5 mg/100 g [105]. The amount estimated in the present study (10.11 to 16.9 mg/100 g) is in agreement with the previous reports (Fig. 35B). DHA content was found to be slightly lower ranging from 8 .67 to 11.67 mg/100 g. No significant change ($p \leq 0.5$) in the ASA or DHA content (Fig. 35C) was observed due to radiation processing or storage in shredded cabbage throughout the storage period of 8 days thus ruling out the possibility of non enzymatic browning in the present case.

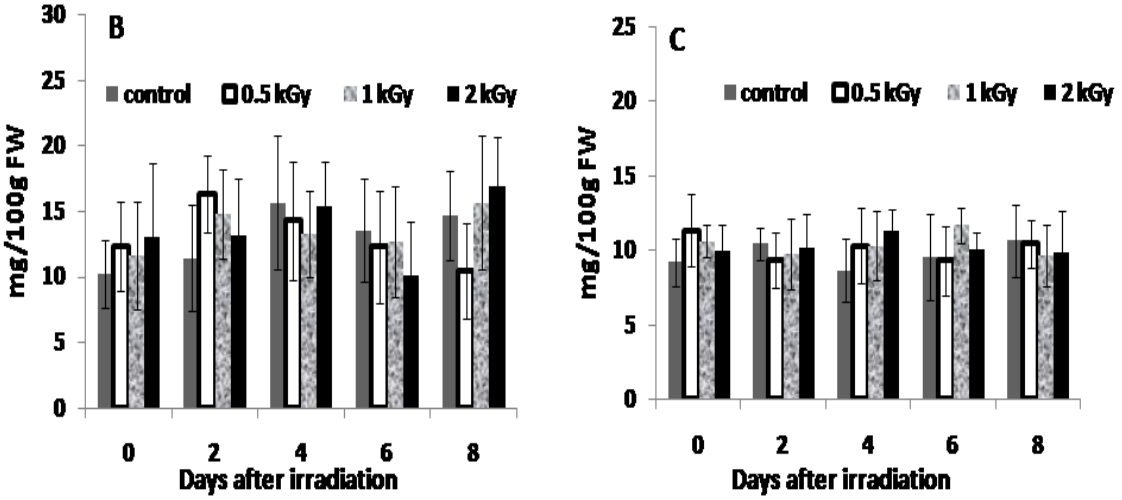


Fig 35 B) Effect of irradiation and storage on ascorbic acid and C) Effect of irradiation and storage on dehydroascorbic acid content in shredded cabbage. Values are expressed as mean ± SD (n=9)

Cut edge browning in shredded cabbage could thus be attributed to the enzymatic reactions in response to wounding following alteration in PAL activity. While some authors have earlier reported γ-irradiation induced browning inhibition in cut vegetables, the present work provides a comprehensive insight on the mechanism of browning inhibition at both biochemical and genetic level for the first time. The current work demonstrated the feasibility of radiation processing as an effective post harvest processing method in inhibiting cut edge browning in shredded cabbage. Thus besides being highly effective method of ensuring food safety γ-irradiation provides an improved benefit in terms of maintaining visual quality of the product.

3.6.4 Role of allyl isothiocyanate in inhibition of browning in shredded cabbage

Allyl isothiocyanate (AITC), one of the key contributors to cabbage aroma has also been reported to impart many bioactive properties to this vegetable. AITC has been associated with anticancer and antimicrobial activity of cabbage. This compound is also reported to have significant hydroxyl radical scavenging, thus contributing to the antioxidant property of the vegetable. Nagata et al [171] have earlier reported the potential role of AITC in inhibiting browning in shredded cabbage. In the present work a significant enhancement in AITC content was noted on irradiation. Hence a possible link between irradiation induced enhancement in AITC and browning inhibition could be inferred.

3.6.4.1 Evaluation of browning

The effect of AITC treatment on cut edge browning in shredded cabbage is shown in Fig 36. The extent of browning was found to decrease with increase in AITC concentration. Cut edge browning was observed in samples treated with 0.005 µl/ml AITC from day 5 onwards. Sample treated with 0.01 µl/ml showed browning at the end of storage period. Interestingly, samples treated with 0.05 µl/ml and 0.1 µl/ml of AITC appeared fresh throughout the storage

period. Thus a dose dependent increase in browning inhibition was obtained for AITC which substantiated the role of this compound as anti-browning agent.

3.6.4.2 Evaluation of enzyme activity

Studies so far have shown that cut edge browning in shredded cabbage was due to the alteration in enzyme activities during storage. The activities of different enzymes (PAL, PPO and POD) treated with various concentrations of AITC were therefore analyzed. Fig 37 illustrates the effect of AITC treatment on PAL activities at different storage periods. Similar to gamma irradiation a concentration dependent decrease in PAL activity was seen in AITC treated samples. Samples treated with 0.005 µl/ml of AITC showed an increase in PAL activity from day 3 onwards while those treated with a concentration of 0.01 µl/ml showed increased PAL activity from day 6 onwards. In 0.05 µl/ml and 0.1 µl/ml treated samples, PAL activity remained unchanged throughout the storage period at all the concentrations studied. PPO and POD activities were also monitored throughout the storage period of 8 days. No change was seen in PPO and POD activity in the control and treated sample throughout the storage period (Table 25). A good correlation (R^2=0.98) was also observed between concentration of AITC and PAL activity (Fig 37 B). Thus, AITC treatment, resulted in a decrease in PAL activity similar to gamma irradiation.

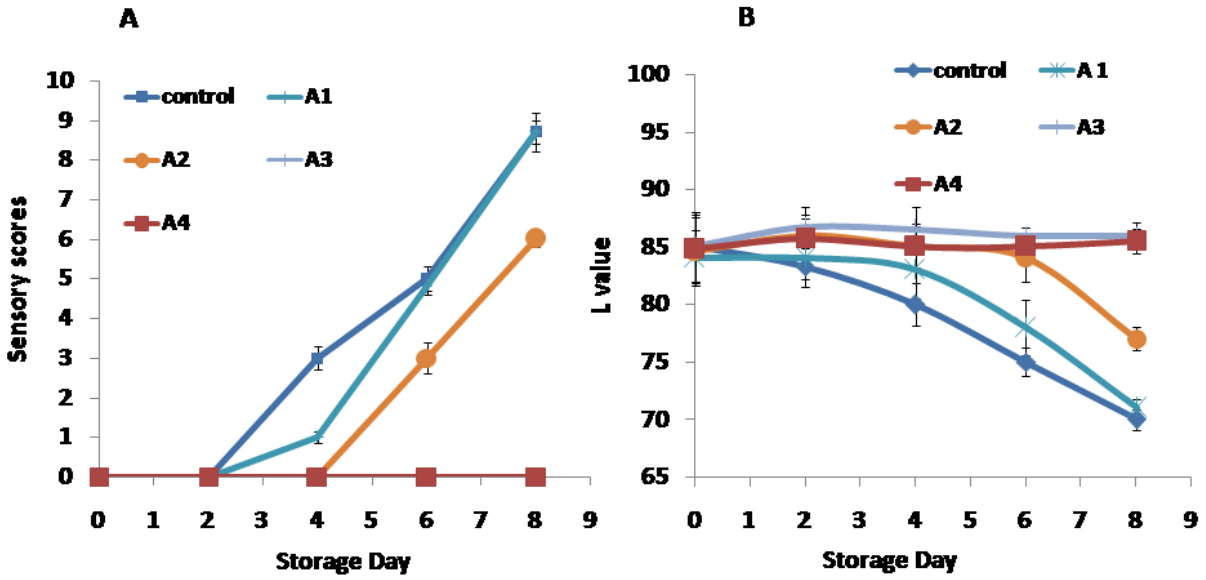


Fig 36 Effect of AITC treatment and storage on cut edge browning in shredded cabbage A) Effect on sensory score for browning B) Effect on L values

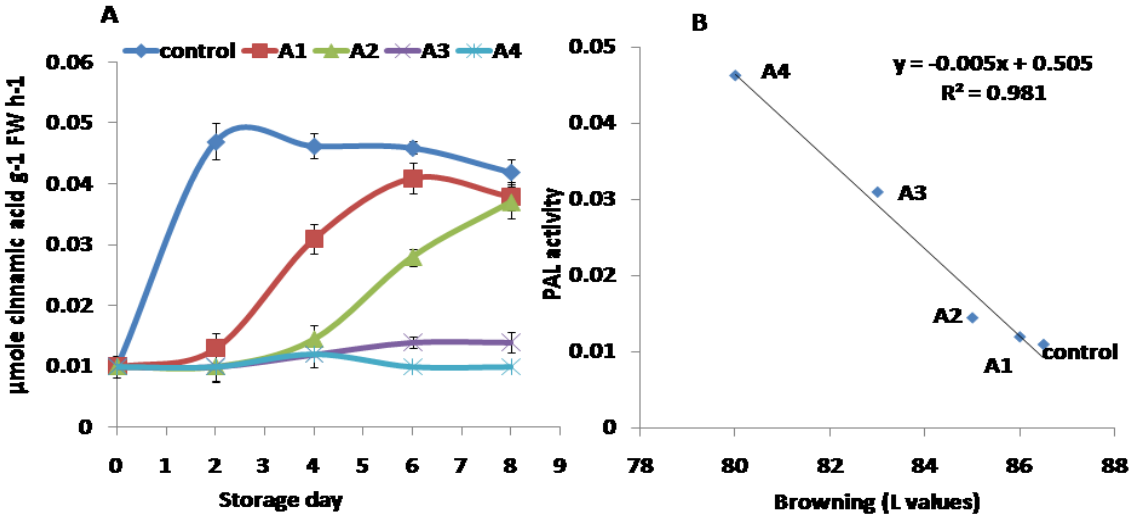


Fig 37A) Effect of AITC treatment and storage (8 days) on PAL activity. B)Plot depicting the relation between PAL activity and browning in shredded cabbage treated with different concentrations of AITC.

Table 25 Effect of AITC treatment on PPO and POD activities

DAY	Control			0.5 kGy			1 kGy			2 kGy		
	PPO	POD1	POD2	PPO	POD1	POD2	PPO	POD1	POD2	PPO	POD1	POD2
0	10.1±2.1[a]	6.1±1.1[a]	5.4±2.2[a]	11.1±2.2[a]	6.9±1.9[a]	4.9±1.8[a]	12.1±4.1[a]	7.1±2.4[a]	5.1±1.1[a]	10.1±3.9[a]	6.9±1.9[a]	4.6±1.1[a]
2	11.1±4.7[a]	7.2±2.2[a]	6.1±1.8[a]	10.7±3.1[a]	6.7±1.7[a]	5.3±1.2[a]	12.3±1.1[a]	6.8±1.8[a]	5.2±1.2[a]	11.1±2.1[a]	5.9±2.1[a]	5.1±2.1[a]
4	12.4±3.1[a]	6.3±1.9[a]	5.1±1.6[a]	12.1±4.2[a]	5.8±2.1[a]	5.9±2.2[a]	10.5±2.8[a]	5.9±1.9[a]	4.9±2.1[a]	12.1±3.3[a]	6.1±2.3[a]	4.9±1.3[a]
6	11.1±2.1[a]	5.8±2.1[a]	4.1±1.4[a]	10.7±2.1[a]	6.1±2.2[a]	6.1±2.3[a]	11.1±4.7[a]	7.1±2.1[a]	5.2±0.7[a]	10.5±3.8[a]	6.7±1.9[a]	6.1±1.5[a]
8	10.2±3.9[a]	6.4±2.3[a]	4.8±2.1[a]	10.9±3.1[a]	6.4±1.2[a]	4.9±1.7[a]	10.9±2.3[a]	6.3±2.2[a]	4.9±1.1[a]	11.1±4.1[a]	5.8±2.1[a]	4.9±1.3[a]

Data are expressed as mean ± standard deviation (n = 9). Mean values in the same column bearing same superscript shows no significant difference ($p \leq 0.05$). PPO activity is represented in U g^{-1}FW, POD activity is represented in Δ A min^{-1} g^{-1} FW; POD1 is caeffic acid peroxidase activity and POD2 is chlorogenic acid peroxidase activity.

3.*6.4.3 Real-time PCR analysis of PAL gene*

Since PAL was the only enzyme being affected by AITC treatment, transcriptional analysis of PAL gene expression at different storage periods was studied. As the minimum concentration required to inhibit cut edge browning for a storage period of 8 days was found to be 0.05 µl/ml, gene expression was analyzed at this concentration of AITC (Fig 38). Gene expression was analyzed at 0, 24 and 48 hrs of storage. Like gamma irradiation, AITC treatment also inhibited browning by decreasing PAL activity. However, the mechanism of this decrease was found to differ. Unlike gamma irradiation where down-regulation in PAL gene expression was seen throughout the storage period, in AITC treated sample a small but significant down-regulation was observed only after 24 hours of storage that reverted back to the basal value by 48 hours thus maintaining constant level of PAL activity in these samples.

3.6.4.3 Total phenolic content and o-quinone content

Total phenolic content decreased significantly on 8^{th} day of storage in samples treated with 0.05 µl/ml and 0.1 µl/ml of AITC. Similar results have been obtained by Wang et al. wherein AITC treatment of blueberries was found to decrease phenolic content on storage [130]. The greater content of phenolics in control samples could be accounted to the increased PAL activity in the these samples. The content of o-quinone was further studied. A concentration dependent decrease in o-quinone content was observed in the AITC treated samples (Fig 39A). The quinone content showed good correlation (R^2=0.99) with PAL activity (Figure 39B) and browning, thus further establishing a direct relationship between increase in PAL activity and browning

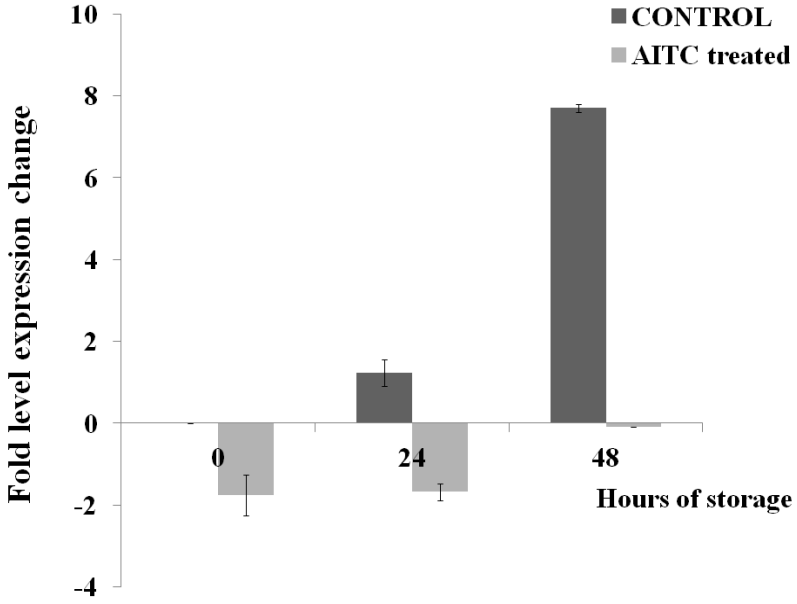


Fig 38 Fold change in the expression of PAL gene on AITC treatment. The x-axis represents the expression level of PAL gene in control at 0 hr. All values are means of triplicates ±SD.

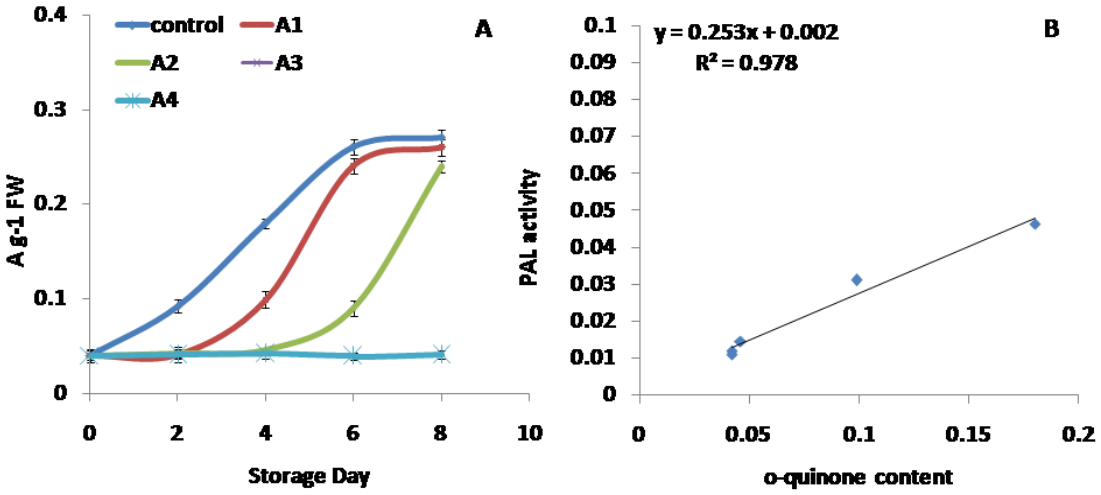


Fig 39 A) Effect of AITC treatment and storage (8 days) on o-quinone content. B)Plot depicting the relation between PAL activity and o-quinone content in shredded cabbage treated with different concentrations of AITC.

Thus, AITC was found to inhibit browning in shredded cabbage by decreasing PAL activity. Although gamma irradiation was found to inhibit browning in a similar manner, transcriptional analysis revealed PAL gene expression to differ in AITC treated sample from that in the irradiated sample. Unlike gamma irradiation where down-regulation in PAL gene expression was seen throughout the storage period, in AITC treated sample down-regulation was observed only at 24 hours of storage that reverted back to the basal value by 48 hours Hence, AITC was found to play no significant role in radiation induced browning inhibition.

3.7 Use of volatile compound for shelf life enhancement of shredded cabbage

Storage of shredded cabbage in an atmosphere of AITC resulted in inhibition of cut edge browning resulting in improvement in the sensory quality of the product. The antimicrobial properties of AITC are well documented. The aroma compounds of plant origin are generally recognized as safe (GRAS) [171,172]. Hence, AITC may prove beneficial in improving shelf life of cut vegetables. However, the use of aroma compounds is often limited due to their impact on the organoleptic characteristics of food products. Use of volatiles which are natural ingredients obtained from food product itself is highly recommendable as they are compatible with the overall oraganoleptic quality of the product. Feasibility of AITC, and other natural volatiles of cabbage namely, *trans*-hex-2-enal, hexanal and hexenyl acetate were studied for shelf life extension of minimally processed shredded cabbage at 10 °C. *trans*-Hex-2-enal was found to be most effective in reducing microbial load followed by AITC, hexanal, and hexenyl acetate. Unlike the other three volatile aroma compounds AITC (0.05 µl/ml) was demonstrated to effectively enhance the sensory quality of the product owing to its antibrowning activity for a storage period of 12 days. Nutritional quality of the product based on total phenolic, flavanoid and vitamin C content as well as the antioxidant properties of the

product was found to be maintained throughout the storage period. AITC was found to effectively increase shelf life of stored minimally processed cabbage at 10 ºC by 5 days maintaining sensory, microbial and nutritional quality of the product. Shredded cabbage when stored in an atmosphere of AITC thus showed a significant increase in its shelf life. AITC being a bactericidal compound maintains the microbial safety of the produce. Also due to its anti-browning activity it improves the sensory quality of the product. Thus the present study has shown that AITC apart from preserving fruits can also effectively increase shelf life of cut vegetables like cabbage. Literature data indicate that aroma compounds can represent a useful tool to increase shelf-life of minimally processed fruits. AITC, 2-hexenal, hexanal, methyl jasmonate and essential oils (eugenol, menthol, thymol etc) have been used to increase the shelf life of fruits like strawberries, apples and blueberries [171]. Shik Shin et al [172] and Ko et al [173] have reported the use of AITC for increasing the shelf life of fermented products like tofu and kimchi. However, studies on the use of these compounds for preservation of minimally processed fresh cut vegetables are limited. A detailed investigation in this direction can prove beneficial to food industry for preservation of fresh cut vegetables.

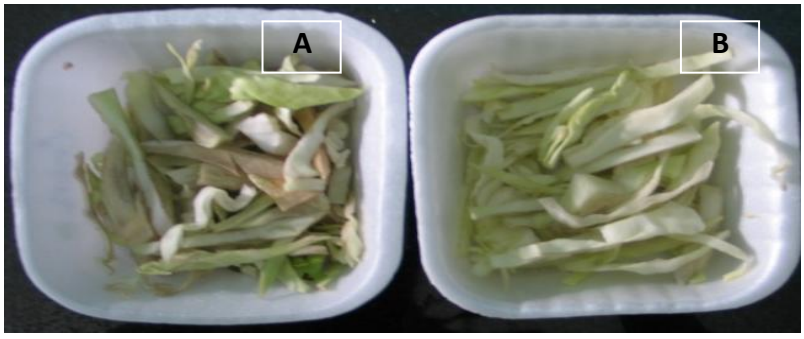


Fig 40 Minimally processed cabbage samples stored at 10 °C after 12 days of storage

A- Control B- AITC treated

Chapter 5

SUMMARY AND CONCLUSION

Vegetables are one of the most important constituents of human diet. The bioactive constituents of vegetables provide various health protective and health promoting properties to consumers. Among the different classes of vegetables, brassica vegetables are one of the most popular species consumed throughout the world. These cruciferous vegetables come with both significant nutritional and health benefits. Additionally, *Brassica* vegetables have increasingly becoming a research model in plant science, as a consequence of the importance of their primary and secondary metabolites. Epidemiological research has shown that consumption of these vegetables is associated with reduced risk of various diseases like cancer and heart diseases. Owing to associated convenience and fresh like characters brassica vegetables are often marketed in minimally processed form. Maintenance of microbial safety along with sensory and nutritional quality of minimally processed vegetables during storage remains the key challenge for food industry. These products are preserved by several methods like refrigeration, chemical preservatives, additives, mild heat treatments, microwave processing, reduction of water activity, ionizing radiation, disinfectants, high pressure technology, high intensity pulsed electric field, pulsed light, ozone technology and hurdle technology. Radiation processing using gamma irradiation is one such technique applied for combating microbial population of fresh produce while maintaining fresh like characters. However, very few reports exist on the impact of radiation processing on the sensory and nutritional quality of brassica vegetables.

Among the different parameters determining the typical quality of brassica vegetables, aroma plays a key role. These cruciferous vegetables have unique aromas attributed to the inherent sulphur compounds particularly the isothiocyanates. Vegetables are also characterized by their green odors. Volatile aliphatic C_6 compounds involving C_6 aldehydes and alcohols and their corresponding hexyl esters, each with their own delicately different odors, decisively contribute to these characteristic odors. These volatile compounds have been found in abundance in most of the green vegetables. Quantitative changes in these volatile constituents produce a green odor distinctive of the plant species. Post harvest

processing operations are known to significantly affect the aroma quality of brassica vegetables thus affecting the consumer acceptability. However, no studies have dealt with the effect of gamma irradiation on aroma profile of brassica vegetables.

Owing to the popularity of brassica vegetables, in the present work four brassica vegetables namely, cabbage, cauliflower, broccoli and radish were chosen for investigation. Preliminary screening was carried out based on the effect of gamma irradiation on odor active compounds. Radiation processing was found to bring about an enhancemnt in AITC, the major isothiocyanate of cabbage. Interestingly no change was seen in aroma profile of any other vegetable. AITC apart from imparting anticancer and antimicrobial properties to cabbage also forms the major aroma contributing compound of this vegetable. Analysis of aroma quality of each of these vegetables by a sensory panel revealed an improvement in aroma quality of irradiated cabbage which could be attributed to the enhancement in AITC content on irradiation. No change was detected in the aroma quality of any other vegetable. Based on above observations cabbage was chosen for further investigation.

The aroma profile three varieties of cabbage (BC-79, NS-22 and market sample) was studied through GC/MS analysis. The aroma profile was predominated by sulfur compounds with AITC being the key compound. Other important compounds being sulfides and GLVs namely, n-hexanal, *trans*-hex-2-enal and *cis*-hex-3-enol. Radiation processing was found to enhance AITC and *trans*-hex-2-enal content in a radiation dose dependent manner. However, the increase was found to be variety dependent. Owing to their bioactive properties these compounds have become a potent candidate of research in food industry. Effect of different processing conditions on aroma compounds of brassica vegetables has been reported. However, to the best of our knowledge this is the first report on gamma irradiation induced enhancement in AITC and GLV content.

GLVs are the products of lipoxygenase pathway of lipid metabolism. Unsaturated fatty acids liberated from galactolipids, phospholipids and triglycerides of plastid membranes have been demonstrated to be

the precursors in the formation of these compounds. Action of lipoxygenase and hydrperoxide lyase on fatty acids generate GLVs. To understand the mechanism of gamma radiation induced GLV enhancement the effect of radiation processing was investigated on lipid profile and associated enzymes of lipoxygenase pathway. MGDG and TAG was noted to be the major lipid in cabbage while TAG, FFA, FAE sterols and PC, PE and PI were found to be the major neutral and phosphoipid respectively. Gamma irradiation was found to decrease MGDG and TAG content in a dose dependent manner in the three varieties of cabbage. A subsequent increase was noted in free fatty acid content. The content of linolenic acid, the precursor of *trans*-hex-2-enal; was found to decrease in a dose dependent manner. Further, the decrease in linolenic acid was found to be linearly correlated ($R^2 = 0.99$) with the increased *trans*-2-hexenal content in the irradiated samples. The increased formation of *trans*-2-hexenal in cabbage immediately after gamma irradiation suggested the possible activation of the enzymes such as acyl hydrolase, lipoxygenase or HPLs. However, no change in the activities of these enzymes were noted at different radiation doses and different storage time, hence ruling out their role in GLV enhancement. Lipid radiolysis and a consequent increased availability of free linolenic acid for the enzymes of lipoxygenase pathway resulted in enhanced *trans*-hex-2-enal in the radiation processed cabbage in the present study.

Apart from GLV, gamma irradiation was found to enhance AITC content in irradiated cabbage. AITC is the hydrolytic product of sinigrin, the major glucosinolate of cabbage. Gamma irradiation induced an enhancement in sinigrin content in irradiated cabbage. Further, a good correlation was also noted between increase in AITC and sinigrin content between different cabbage varieties. Thus the increased AITC observed in the steam distilled volatile oils from radiation processed vegetable could be the result of hydrolytic breakdown of more available sinigrin in the treated samples. To the best of our knowledge this is the first report on gamma radiation induced enhancement in glucosinolate, the potent anticancerous compounds reported in Brassica vegetable.

A number of environmental conditions can enhance glucosinolate content significantly. Selective induction of glucosinolates can be brought about by jasmonates. Interestingly, these molecules are byproducts of lipoxygenase pathway and are derived from linolenic acid. Hence a correlation between linolenic acid enhancement during radiation processing and jasmonate was speculated. However, no change in the content of these signalling molecules was noted in samples subjected to different radiation doses at different storage periods thus ruling out the role of these compounds in glucosinolate enhancement.

Mechanism of radiation induced glucosinolate enhancement was further investigated at molecular level. Transcriptional analysis of genes associated with glucosinolate biosynthesis revealed a modulation in their expression on exposure to gamma irradiation. *MYB28, CYP79F1, CYP83A1* and *SUR1* were found to be up-regulated resulting in an enhancement in content of aliphatic glucosinolate. Hence increase in GLS content was accounted solely to be the effect of gamma irradiation without intervention of any other signalling molecule. To the best of our knowledge this is the first report on effect of gamma irradiation on glucosinolate biosynthesis genes. *MYB28* is the transcription factor regulating the expression of *CYP79F1* and *CYP83A1* genes. Hence, the gamma irradiation induced increase in *MYB28* gene expression could lead to an increase in expression of *CYP79F1* and *CYP83A1*. *CYP79F1* and *CYP83A1* are the genes encoding enzymes for synthesis of glucosinolate core structure, hence, an increase in their expression can directly increase the glucosinolate content in irradiated vegetable.

In the present study gamma irradiation was demonstrated to improve the sensory quality of cabbage by improving its aroma quality. Use of radiation processing for microbial decontamination is well documented. Hence it was of interest to investigate the feasibility of radiation processing to develop minimally processed ready to cook cabbage with improved sensory quality. Gamma irradiation (0.5-2.5 kGy) in combination with low temperature (4-15 °C) storage was attempted to increase shelf life of cabbage wrapped in cling films. A maximum extension in shelf life of 8 days, while retaining its

sensory and microbial quality, was achieved when the product was irradiated to 2 kGy and stored at 10 °C. Gamma irradiation also inhibited browning at their cut edges resulting in improved visual appeal. An increase in total antioxidant activity was observed with respect to DPPH and hydroxyl radical scavenging ability while the ferric reducing property and nitric oxide radical scavenging activity remained unaffected. Total phenolic, flavonoid and vitamin C content remained unchanged due to irradiation. Gamma-radiation induced inhibition of browning in minimally processed shredded cabbage stored (10°C) up to 8 days was further investigated. In the non-irradiated samples, phenylalanine ammonia lyase (PAL) activity increased during storage that could be linearly correlated with enhanced quonine formation and browning. No significant change was observed in polyphenol oxidase, peroxidase and in the total as well as individual phenolic content in both non-irradiated and irradiated samples. Transcriptional analysis showed an up-regulation in PAL gene expression in the non-irradiated stored samples. Gamma irradiation (2kGy), however resulted in inhibition of browning as a result of down-regulation in PAL gene expression and a consequent decrease in PAL activity. The present work is the first report on the mechanism of gamma irradiation induced browning inhibition in cut vegetables. AITC has been reported to be a potent inhibitor of browning. Owing to radiation induced enhancement in AITC content, a correlation was speculated between AITC enhancement and browning inhibition in irradiated cabbage. Similar to radiation processing AITC treatment was found to inhibit browning in shredded cabbage by reducing PAL activity however transcriptional analysis of PAL gene expression showed the expression pattern to vary from radiation induced PAL downregulation thus ruling out the role of AITC in radiation induced browning inhibition.

In the view of antibrowning and bactericidal activity of AITC, AITC and few other natural volatiles of cabbage namely, *trans*-hex-2-enal, hexanal and hexenyl acetate were applied for shelf life extension of minimally processed shredded cabbage. A shelf life enhancement of 5 days was found with AITC while maintaining microbial, sensory and nutritional quality of treated samples.

Based on these studies on gamma irradiation on phytochemical constituents of cabbage the following important conclusions were drawn:

1) Gamma irradiation enhanced the aroma quality of irradiated cabbage owing to an increase in AITC and GLV content.

2) Lipid radiolysis by gamma irradiation caused an enhancement in free fatty acid pool. Released linolenic acid was acted upon by enzymes of LOX pathway enhancing 2-hexenal content.

3) Gamma irradiation upregulated expression of genes of glucosinolate biosynthesis resulting in an increase in glucosinolate content. The increase in glucosinolate content accounted for an enhancement in AITC content.

4) Gamma irradiation enhanced the shelf life of RTC cabbage at 10 °C by 8 days while maintaining microbial, sensory and nutritional quality of the product.

5) Sensory quality was enhanced as a result of enhanced aroma and visual quality of the product. Gamma irradiation resulted in a down regulation of PAL gene expression causing a reduction of PAL gene activity thus inhibiting browning in these samples.

6) Radiation processing was thus demonstrated to enhance the aroma quality of cabbage by increasing GLV and AITC content. Enhanced formation of GLVs was directly correlated to lipid metabolism. The pathway involved in enhancement in glucosinolate content was however found to be jasmonate independent and thus could not be correlated with lipid metabolism.

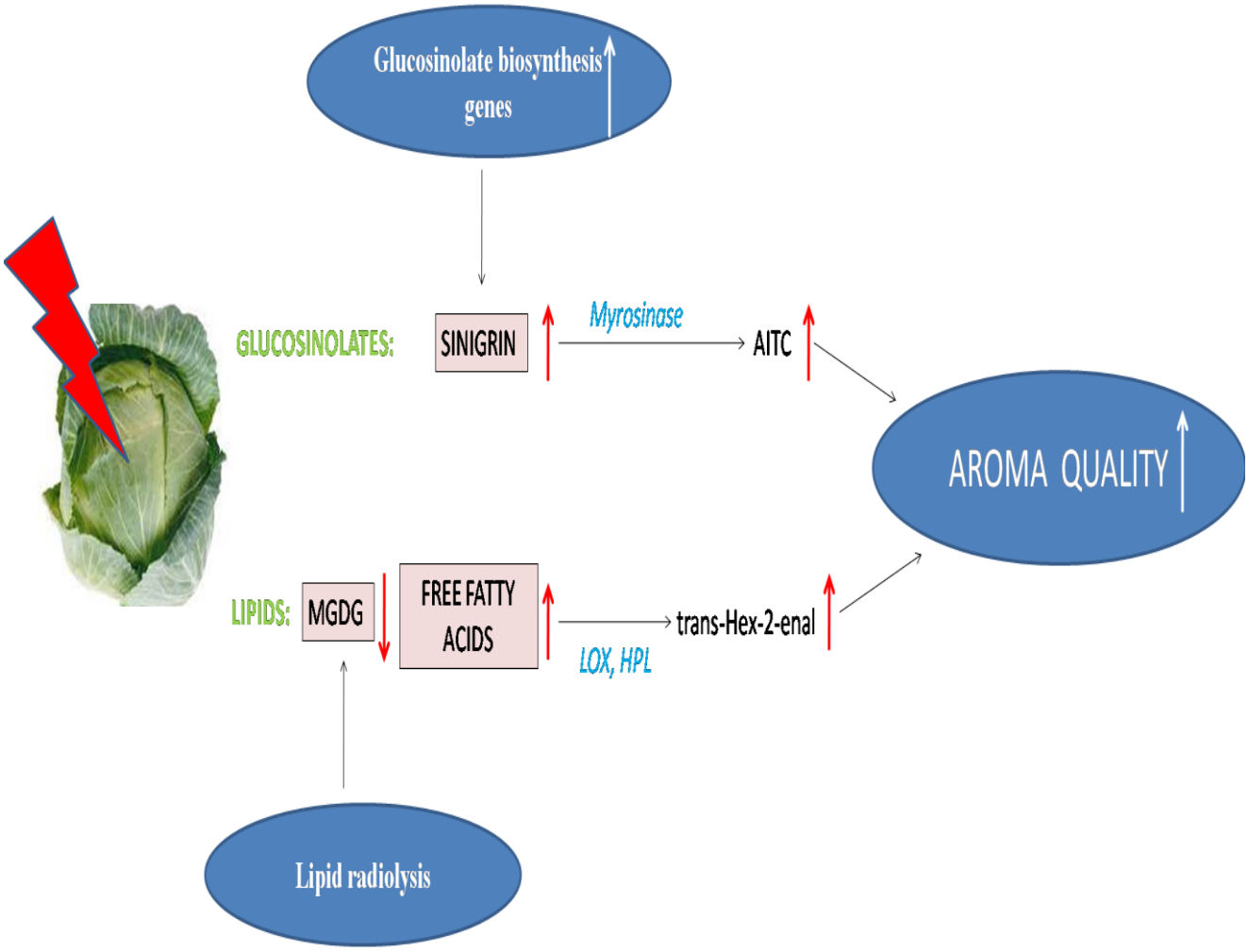


Fig 41 Diagramamatic representation of the impact of gamma irradiation on aroma quality of cabbage.

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1. Banerjee, A., Variyar, P. S., Chatterjee, S., & Sharma, A. (2014). Effect of post harvest radiation processing and storage on the volatile oil composition and glucosinolate profile of cabbage. *Food Chemistry,* 151, 22-30.

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b. Under Review:

1. Banerjee, A., Chatterjee, S., Variyar, P. S., & Sharma, A. Shelf life extension of minimally processed ready to cook (RTC) cabbage by gamma irradiation. Journal of Food Science and Technology.

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Effect of post harvest radiation processing and storage on the volatile oil composition and glucosinolate profile of cabbage

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ABSTRACT

Effect of radiation processing (0.5–2 kGy) and storage on the volatile oil constituents and glucosinolate profile of cabbage was investigated. Among the volatile oil constituents, an enhancement in *trans*-hex-2-enal was noted on irradiation that was attributed to the increased liberation of precursor linolenic acid mainly from monogalactosyl diacyl glycerol (MGDG). Irradiation also enhanced sinigrin, the major glucosinolate of cabbage that accounted for the enhanced allyl isothiocyanate (AITC) in the volatile oils of the irradiated vegetable. During storage the content of *trans*-hex-2-enal increased immediately after irradiation and then returned to the basal value within 24 h while the content of sinigrin and AITC increased post irradiation and thereafter remained constant during storage. Our findings on the enhancement in potentially important health promoting compounds such as sinigrin and AITC demonstrates that besides extending shelf life and safety, radiation processing can have an additional advantage in improving the nutritional quality of cabbage.

1. Introduction

Leaves of *Brassicaceae* family are recognised for their nutritional value and are familiar components of salads around the world. Fresh leaves of cabbage (*Brassica oleracea*), a vegetable of the *Brassica* family are used for preparation of a wide variety of recipes including delicacies like sauerkraut and kimchi. They possess a typical flavor and odor attributed to volatile sulfur compounds (Eskin, 2012). Isothiocyanates have been shown to be the major compounds that impart pungent flavor and sulfurous aroma to these vegetables. Cruciferous vegetables including cabbage have also been extensively investigated recently for their contribution to the anticarcinogenic compounds in the diet (Traka & Mithen, 2009). Isothiocyanates have been reported to be mainly responsible for the observed chemoprotective activity of these vegetables (Traka & Mithen, 2009). The isothiocyanates are the hydrolytic products of sulfur containing glucosides namely glucosinolates. Cleavage of the glucose moiety from glucosinolates by enzyme myrosinase in the presence of water results in an unstable aglycone that gets converted to a thiocyanate, an isothiocyanate or a nitrile (Traka & Mithen, 2009). These hydrolytic products are the active substances produced by plant as defence against pathogens. Due to their anticarcinogenic properties, glucosinolates and their hydrolysed products have generated considerable interest as nutraceuticals. Fresh vegetables possess a green odor that also contributes to their organoleptic quality. These odors are attributed to the release of C_6 aldehydes and alcohols and their corresponding esters, collectively termed as green leaf volatiles (GLVs) (Hatanaka, 1996). Unsaturated fatty acids liberated from galactolipids, phospholipids and triglycerides of plastid membranes have been demonstrated to be the precursors in the formation of these compounds (Hatanaka, 1996). A group of lipid hydrolysing enzyme called lipases release fatty acids from the membrane lipids. The released fatty acids are acted upon by enzymes such as a non-heme iron dioxygenase called lipoxygenase (LOX) and further by hydroperoxide lyase (HPL) of the lipoxygenase pathway to form C6 aldehydes and alcohols (Hatanaka, 1996).

Widespread outbreak of food borne illness worldwide in recent years has been associated to the consumption of fresh leafy vegetables. Traditional methods for elimination of food borne pathogens from these vegetables such as blanching and mild heat treatment can result in lowering their sensory quality. This necessitates the use of non thermal methods for reducing health risks. In this regard, use of radiation processing for elimination of food borne pathogens as a viable alternative, while maintaining fresh attributes of the produce, has been recognised (Arvanitoyannis, 2010; Arvanitoyannis, Stratakos, & Tsarouhas, 2009). However, the effect of such a processing on the flavor, aroma and bioactive constituents of cruciferous vegetables has not been extensively investigated. We report here the effect of radiation processing at recommended doses on the content of GLVs and isothiocyanates in cabbage. The impact of radiation processing on the enzymes of the lipoxygenase pathway and on the content of GLV as well as

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on isothiocyanate precursor, glucosinolates, is of specific interest and will be investigated.

2. Materials and methods

2.1. Materials

Cabbage (*B. oleracea*) samples of BC-79 and NS-22 varieties were obtained from farmers of Akola district, Maharashtra, India. The samples were authenticated at Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola as belonging to the above varieties. Harvesting was done 65 days after planting when the vegetable was known to be mature. A variety of unknown origin was also obtained from a local market in Mumbai for comparison and was designated as market sample.

Chemicals were purchased from various suppliers: *trans*-hex-2-enal, sinigrin, tripalmitylglycerol, linoleic acid and linolenic acid from Sigma–Aldrich (USA); allyl isothiocyanate from Fluka, Sigma–Aldrich (USA); Monogalactosyldiacylglycerol (MGDG), Digalactosyldiacylglycerol (DGDG) from Avanti polar lipids (India); lipoxygenase, and sulfatase from Sigma–Aldrich (USA). All solvents were procured from Merck (India) and redistilled before use.

2.2. Irradiation of cabbage samples

Cabbage samples were subjected to gamma irradiation using a ^{60}Co gamma irradiator (GC-5000, BRIT, India, dose rate 4.1 kGy/h) in air to an average absorbed dose of 0.5, 1, and 2 kGy. Dosimetry was carried out using Fricke dosimeter.

2.3. Simultaneous steam distillation extraction and GC–MS analysis

Blended cabbage leaves (200 g) were subjected to steam distillation using simultaneous distillation–extraction technique as described earlier (Variyar, Ahmad, Bhat, Niyas, & Sharma, 2003). The essential oils (mg/wet weight) thus obtained were then subjected to GC–MS analysis using similar parameters as described earlier (Variyar et al., 2003). Peaks were identified by comparing their mass fragmentation pattern (Wiley/NIST Libraries), retention time and Kovats index with standards. The amount of each individual compound present in the sample was calculated by mean of the internal standard, and expressed as mg per kg of dry weight.

2.4. Extraction and analyses of lipids

Cabbage leaves (300 g), ground in liquid nitrogen were extracted in 900 mL of chloroform: methanol (2:1) as reported earlier (Chatterjee, Variyar, & Sharma, 2010). The total lipid extract thus obtained was subjected to silica gel TLC (Kieselgel 60, Merck, Germany). Neutral lipids were analysed using solvent mixture of hexane:diethyl ether:acetic acid (80:20:2) while phospholipids were separated and identified using ethyl acetate:2-propanol: chloroform:methanol:0.25% aq KCl (25:25:25:10:9) as the developing solvent system. Separation of galactolipids was carried out using chloroform: methanol: water (80:18:2) as the solvent system. The individual lipid class was identified from R_f values of standards spotted separately on the same plate. The separated spots were visualised by exposing to iodine vapor and the area of the individual spots was quantified on a TLC-densitometer (CS9301PC, Shimadzu, Japan) from a standard curve of spot area vs. concentration using different concentrations of standard lipid species referred above. Free fatty acids were isolated using 50 mg of lipid extract containing dodecanoic acid (50 μg) as internal standard and analysed by GC/MS after converting to methyl esters using diazomethane under similar parameters (Chatterjee et al., 2010). To analyse fatty acid composition of MGDG and TAG, total lipid extracts were subjected to preparative (0.5 mm thickness) silica gel TLC using solvent system used for neutral lipid and galactolipid separation. The bands corresponding to TAG and MGDG were isolated, hydrolysed, methylated with diazomethane and subjected to GC–MS.

2.5. Lipase assay

Cabbage leaves (20 g) were extracted in 60 mL of ice cold extraction buffer (0.1 M TrisHCl, pH 8) containing 0.1 M KCl, 0.1% Triton X-100 and 2 g PVPP as reported earlier (Pérez, Sanz, Olías, & Olías, 1999). Lipase activity was measured by quantifying spectrophotometrically (410 nm) the *p*-nitrophenol (λ_{max} 410) released following hydrolysis of *p*-nitrophenyl laurate substrate by lipase as described previously (Pisirodom & Parkin, 2001). Reaction was started by the addition of 1 mL enzyme extract to 2.5 mL 420 μM *p*-nitrophenyl laurate in 2.5 mL Tris–HCl buffer (0.1 M, pH 8.2). Absorbance was monitored in a spectrophotometer (UV-2450, Shimadzu, Japan) up to 15 min. p-Nitrophenol standard curve was used to convert absorbance to μM substrate hydrolysed.

2.6. Lipoxygenase assay

Crude enzyme was extracted in sodium phosphate buffer according to Gardner (2001). Lipoxygenase activity was measured as conjugated diene formed (Gardner, 2001). The reaction mixture contained linoleic acid (7.5 mM, 10 μl) and 30 μl crude extract made up to volume (3 mL) with 0.1 M acetate buffer (pH 5). Absorbance was measured for 10 min using a spectrophotometer. An extinction coefficient of 25,000 $M^{-1}cm^{-1}$ was used to convert absorbance values at 234 nm to μmol of conjugated diene.

2.7. Hydroperoxide lyase assay

Extraction procedure followed was same as for lipase assay. HPL was assayed by the loss in absorption at 234 nm by the hydroperoxide (Vick & Zimmerman, 1976). Briefly, linoleic acid substrate (0.6 mL of 7.5 mM) was incubated with 1.12 mg of soybean lipoxygenase (100,000 units/mg) in 30 mL distilled water for 1 h to obtain a solution of hydroperoxide substrate. The final reaction mixture contained 250 μl of the hydroperoxide substrate prepared earlier and 250 μl of enzyme solution made up to a volume of 3 mL with potassium phosphate buffer (0.1 M, pH 6). Readings were taken for 10 min by a spectrophotometer. An extinction coefficient of 25,000 M^{-1} cm^{-1} was used to convert absorbance values at 234 nm to μmol of products formed.

2.8. Analysis of end products of lipid oxidation

Cabbage sample (30 g) was blended in a homogenizer with 100 mL of ice cold phosphate buffer (50 mM, pH 7), containing 0.2 mM EDTA, 0.2% TritonX-100 and 2 g PVPP. Resulting homogenate was vacuum filtered and the residue washed 2 times with 25 mL of buffer. Extracts were centrifuged at 14,000 rpm for 20 min at 4 °C. To 2 mL of supernatant, 2 mL of 10 mM acid substrate (linolenic or linoleic acid) in 10 mL phosphate buffer (100 mM, pH 7) was added and incubated for 30 min. Reaction was stopped by adjusting pH to 3. The mixture was passed through a C18 extraction column (Superclean ENVI-18 SPE, 500 mg) and the products eluted with methanol. The residue after removal of methanol was esterified with 2 M methanolic KOH and subjected to GC–MS analysis.

2.9. Isolation of glucosinolates

Glucosinolates were isolated from freeze dried cabbage leaves (5 g) using 100 mL of boiling water containing glucotropaeolin (100 μl, 20 mM) as internal standard for 10 min as reported earlier (Kaushik & Agnihotri 1999). The crude aqueous extract thus obtained was successively extracted with ethyl acetate (3 × 30 mL) and *n*-butanol (3 × 30 mL). The butanol extract was concentrated to dryness to obtain 10% solution.

2.10. Identification of sinigrin

The butanol extract was subjected to HPLC (Jasco HPLC system, Japan) using 50 mM ammonium acetate as solvent A and 50 mM ammonium acetate: methanol (80:20) as solvent B using RP C-18 (HYPERSIL, Chromato-pack, Mumbai, India) column (250 mm × 4.6 mm, 10 μ), solvent gradient as time 0 min, A = 100%; time 40 min, A = 0% at a flow rate of 0.3 mL/min, at 235 nm wavelength. Sinigrin, the major glucosinolate in cabbage, was identified by comparing its R_t with that of the standard compound and from its mass fragmentation pattern on an LC/MS instrument. Mass spectra were recorded by atmospheric pressure chemical ionisation in the negative mode using a Varian Ion Trap MS (410 Prostar Binary LC with 500 MS IT PDA detectors) equipped with a C-18 reverse phase stainless steel column (30 cm × 0.46 cm). All samples were filtered through a 0.45 μm filter (Millipore Corp.) before injection. The capillary voltage was kept at 80 V, and the air (nebulizing gas) pressure was 35 psi. Full scan data acquisition was performed by scanning from m/z 100 to 900.

Glucosinolates were desulfated using 10 mL crude aqueous extract (10% solution) to which 500 μl of 0.02 M sulfatase enzyme in aq NaAcO–AcOH (pH 5) was added and incubated overnight. The resultant mixture was subjected to HPLC analysis as above for further identification of sinigrin.

2.11. Myrosinase assay

40 g of cabbage leaves were blended in a homogenizer with 100 mL cold sodium phosphate buffer of (33 mM, pH 7) containing 0.2 M NaCl. The resulting suspension was centrifuged at 14,000 rpm at 4 °C for 20 min and the supernatant was used for myrosinase activity determination. Myrosinase activity was assayed based on the loss in absorption at 235 nm resulting from hydrolysis of allyl glucosinolate. Briefly, 0.2 mM sinigrin, 500 μM ascorbic acid and 1 mM EDTA were incubated at 37 °C. Reaction was started by addition of 50 μl of supernatant. Readings were taken for 10 min by a spectrophotometer. An extinction coefficient of 6780 M^{-1} cm^{-1} was used to convert absorbance values at 235 nm to μmol of products formed.

2.12. Statistical analysis

DSAASTAT ver. 1.101 by Andrea Onofri was used for statistical analysis of data. Data was analysed by Analysis of variance (ANOVA) and multiple comparisons of means were carried out using Duncan's multiple range test. Data are expressed as means ± SD of three independent analyses each carried out in triplicate. Means are expressed as significantly different or not at 5% level of confidence.

3. Results and discussion

3.1. Volatile oil composition

Table 1 lists the major volatile compounds identified in different varieties of cabbage. The nature of the compounds identified is similar to that reported in literature (Eskin, 2012). Qualitative and quantitative differences in the volatile constituents were noted between the varieties currently investigated. The content and pattern of volatiles are reported to vary according to plant species, cultivars and vegetable part, as well as with the developmental stage of the plant (Eskin, 2012). Allyl isothiocyanate (AITC) was the major compound identified in all the varieties. This compound, derived by the hydrolytic cleavage of the glucosinolate, sinigrin, and possessing a sulfurous, garlic and pungent odor is known to impart characteristic odor to cabbage. The compound showed a wide variation in its distribution among the different varieties (Table 1). The market sample had the highest content of AITC, followed by BC-79 and NS-22. Variation in this major isothiocyanate among the different varieties can have a significant impact on their flavour quality. Other isothiocyantes identified such as but-3-enyl isothiocyanate and 3-(methylthio) propyl isothiocyanates, expected to be derived from gluconapin and glucoibervirin respectively, also showed a wide variation in their distribution among the different varieties. Their content was highest in market samples and lowest in NS-22. However, the impact of changes in these minor isothiocyanates on the overall odor quality of the vegetable can be assumed to be insignificant due to their far lower concentrations in the vegetable and higher odor threshold compared to AITC. Volatile sulfides such as dimethyl disulfide (DMDS) with a sulfurous cabbage like odor, dimethyl trisulfide (DMTS) with a sulfurous cauliflower like odor and dimethyl tetrasulfide (DMTES) having a garlic meaty odor are known to be odor active compounds of cooked Brassica species. These compounds are derived either from (+)-*S*-methyl-L-cystein sulfoxide found in Brassica vegetables or formed by degradation of volatiles derived from glucosinolate break down. The content of these volatile sulfides also varied significantly among the three varieties (Table 1). A wide variation in the content of volatile sulfur compounds among cultivars and with the maturity has been reported in *Brassica* species. Changes in the distribution of volatile sulfur compounds identified can have a significant impact on the aroma quality of the vegetable.

Other compounds present in significant amounts include *n*-hexanal, *trans*-hex-2-enal and *cis*-hex-3-enol. These compounds with a characteristic green odor are associated with sensory perception of freshness (Hatanaka, 1996). Hexanal, characterised by green, grassy odor note, is reported to be the key odor compound of fresh broccoli florets while *trans*-hex-2-enal and *cis*-hex-3-enol, possessing fresh green and leafy aroma are reported to play a relatively important role in cabbage flavour (Eskin, 2012). The above C6 aldehydes and alcohols that form part of the GLVs are known to be formed via the lipoxygense pathway from unsaturated fatty acid precursors namely linoleic and linolenic acids liberated mainly from galactolipids. The content of *n*-hexanal and *trans*-hex-2-enal was highest in the market samples. The concentration of these two compounds was, however, higher in NS-22 than BC-79. No significant differences in the distribution of *cis*-hex-3-enol was noted between the different varieties tested. C6 aldehydes formation in leaves has also been reported to be under developmental control and therefore dependent on leaf age. The changes in the distribution of GLVs observed could thus be possibly explained by the variation in the maturity between the various varieties studied.

3.2. Effect of radiation processing on volatile constituents

Processing by high energy ionising radiation is an important post-harvest preservation technique currently practiced worldwide. The process has recently been recommended for microbial decontamination of fresh leafy green vegetables of the Brassica species such as spinach and lettuce. FDA USA (http://www.fda.gov/ForConsumers/ConsumerUpdates/ucm093651.htm)

Table 1
Effect of irradiation (2 kGy) on volatile oil composition for 3 different varieties of cabbage.

Volatiles	KI	NS-22 (mg/kg)		BC-79 (mg/kg)		Market sample (mg/kg)	
		Control	Irradiated	Control	Irradiated	Control	Irradiated
Dimethyl disulfide (methyldisulfanyl methane)	536	0.21 ± 0.03[a]	0.28 ± 0.09[a]	1.71 ± 0.09[a]	1.78 ± 0.19[a]	1.47 ± 0.09[a]	1.27 ± 0.61[a]
2-Penten-1-ol	686	0.049 ± 0.007[a]	0.047 ± 0.01[a]	0.07 ± 0.03[a]	0.07 ± 0.06[a]	–	–
n-Hexanal	801	1.66 ± 0.09[a]	1.62 ± 0.09[a]	0.39 ± 0.03[a]	0.39 ± 0.07[a]	2.09 ± 0.1[a]	1. 98 ± 0.8[a]
trans-Hex-2-enal	849	1.1 ± 0.11[a]	1.96 ± 0.16[b]	0.64 ± 0.02[a]	0.90 ± 0.02[b]	1.18 ± 0.23[a]	2.01 ± 0.15[b]
cis-Hex-3-enol	858	2.6 ± 0.54[a]	2.1 ± 0.89[a]	2.3 ± 0.32[a]	2.9 ± 0.12[a]	2.77 ± 0.53[a]	2.48 ± 0.65[a]
Allyl isothiocyanate (3-isothiocyanato-prop-1-ene)	887	9.4 ± 1.83[a]	16.89 ± 3[b]	28.3 ± 1.9[a]	40.9 ± 1.61[b]	31.5 ± 3.11[a]	52.92 ± 6.5[b]
Dimethyl trisulfide (methyltrisulfanyl methane)	974	1.66 ± 0.09[a]	1. 6 ± 0.19[a]	14.5 ± 2.61[a]	13.6 ± 1.76[a]	4.22 ± 0.99[a]	3.92 ± 1.01[a]
But-3-enyl isothiocyanate (4-isothiocyanato-but-1-ene)	1100	1.63 ± 0.33[a]	1.89 ± 0.43[a]	2.26 ± 1.09[a]	2.06 ± 0.98[a]	3.01 ± 0.73[a]	2.9 ± 0.8[a]
Dimethyl tetrasulfide (methyldisulfanyldisulfanyl methane)	1232	0.11 ± 0.005[a]	0.11 ± 0.06[a]	0.98 ± 0.19[a]	0.93 ± 0.17[a]	0.43 ± 0.05[a]	0.39 ± 0.08[a]
3-(Methylthio)propyl isothiocyanates (1-isothiocyanato-3-methylsulfanyl propane)	1290	0.007 ± 0.007[a]	0.0074 ± .005[a]	0.34 ± 0.09[a]	0.38 ± 0.1[a]	1.02 ± 0.09[a]	0.91 ± 0.19[a]

Data are expressed as mean ± standard deviation ($n = 9$). Mean values in the same column for a variety bearing different superscripts are significantly different ($p < 0.05$).

allows the use of ionising radiation up to 4 kGy to make these products safer and delay spoilage. Arvanitoyannis (2010) have recently published an exhaustive review on the irradiation applications in vegetables and fruits. No published literature, however, exists on the effects of such a processing method on the content of glucosinolates, their hydrolytic products and GLVs in vegetables of the Brassica species. In order to determine the optimum dose that could be allowed for treatment of cabbage leaves the effect of radiation processing at various doses on the sensory acceptability was initially investigated by a trained panel (data not shown). The sensory panel could clearly detect off odors at doses beyond 2.5 kGy and hence the volatile oils isolated from the vegetable exposed to doses beyond 2 kGy were not investigated. Various reports exist on the shelf life extension of vegetables at doses between 1 and 2 kGy (Arvanitoyannis, 2010). Irradiation was found to have no effect on the quality parameters of cabbage up to a dose of 1 kGy (Arvanitoyannis, 2010). Table 1 shows the effect of radiation processing at 2 kGy on the composition of the volatile oil obtained from different varieties of cabbage. Except for AITC and *trans*-hex-2-enal, the content of other constituents identified were unaffected by radiation processing. An increase in the content of AITC was noted immediately after irradiation in all the varieties studied. The extent of this increase differed depending on the variety. The highest increase was noted for NS-22 with an increase in content by 80% followed by the market sample (68%) and variety BC-79 (44.5%). Fig. 1A depicts the effect of various doses (0.5–2 kGy) on the formation of AITC in NS-22 variety. An increase in the content of AITC with dose was noted. The increase in AITC content was not found to be significantly affected by post-irradiation storage (10 °C) with a slight decrease in the content during storage (Fig. 1B). The content of this compound was, however, considerably higher than the control samples throughout the storage period studied. To the best of our knowledge this is the first report on the gamma radiation induced enhancement in AITC content in cabbage. As AITC is known to contribute to the characteristic odor and taste of the vegetable, its enhanced formation during radiation processing can have a significant impact on its flavour quality. The content of *trans*-hex-2-enal increased significantly immediately after irradiation while that of *n*-hexanal and *cis*-hex-3-enol was unaffected. The increase in *trans*-hex-2-enal was found to be variety dependent with the highest increase noted in NS-22 (78%) followed by the market sample (70.3%) and BC-79 (40.6%). *trans*-Hex-2-enal is known to be an oxidative product of linolenic acid formed via the lipoxygenase pathway (Hatanaka, 1996). Variation in the content of linolenic acid liberated as a result of lipid radiolysis in the different varieties could possibly explain the observed variation in *trans*-hex-2-enal content. Byun, Kang, Kwon, Hayashi, and Morf (1996) reported an increased *n*-hexanal content in soybeans due to gamma irradiation at a dose above 10 kGy with as high as 5 times increase at 100 kGy. Fan and Sokorai (2002) on the other hand observed an increase in *trans*-hex-2-enal content of cilantro during post-harvest storage with no significant effect on the content of this compound on irradiation. Fig. 1A depicts the effect of radiation processing at various doses on the content of *trans*-hex-2-enal in NS-22 cabbage variety. Similar to AITC, an increase in content of *trans*-hex-2-enal with dose was noted. The content of *trans*-hex-2-enal formed was also found to vary with post-irradiation storage. A rapid decrease in *trans*-hex-2-enal content of the irradiated vegetable from its initial value was noted on storage up to a period of one day beyond which its concentration was comparable to that of the non-irradiated sample (Fig. 1B). GLVs are known to be released almost immediately after wounding (Matsui, 2006). Their content has been shown to decrease within few hours after wounding and thus can be considered as typical wound signals. As irradiation was not found to affect the content of other volatiles identified in the present study, the mechanism of increased AITC and *trans*-hex-2-enal was further investigated.

3.3. Lipid composition

Wound-induced volatile compounds, such as aldehydes, especially *trans*-hex-2-enal, alcohols, and esters are biologically active compounds derived from the LOX via polyunsaturated fatty acids such as linoleic and linolenic acids. The nature of the lipid and the fatty acid composition was therefore of interest. Fig. S1 depicts the TLC chromatograms of neutral and galactolipid species in the control and irradiated (0.5–2 kGy) cabbage. Fatty acid esters, triacylglycerol (TAG), free fatty acids (FFA) and sterols were identified as the major neutral lipid constituents while MGDG and DGDG were the predominant galactolipid components identified in cabbage. The major phospholipids identified include phosphatidylethanolamine, phosphatidylinositol, and phosphatidylcholine (data not shown). Triacylglycerols are reported to be the main constituents in Brassica oils. In an earlier work on the lipid composition of cabbage oil, Peng (1974) reported the presence of neutral lipids, glycolipids and phospholipids as the major lipid constituents of the vegetable accounting for 51.02%, 40.78% and 8.18% of the oil. The distribution of various lipid species identified in the present study is comparable to the reported literature values. The content of the lipid constituents varied considerably depending on the variety (Table 2). BC-79 variety had the highest TAG, fatty acid ester and galactolipid (MGDG and DGDG) content. On the other hand, the content of FFA and sterols as well as phospholipids identified was highest in the market samples. Variations in these lipid species can have a significant impact on the content of precursor fatty acids liberated during radiation processing and thus on the content of GLVs in the volatile profile of the vegetable. Table 2 also lists the nature of fatty acids and their content in different cabbage

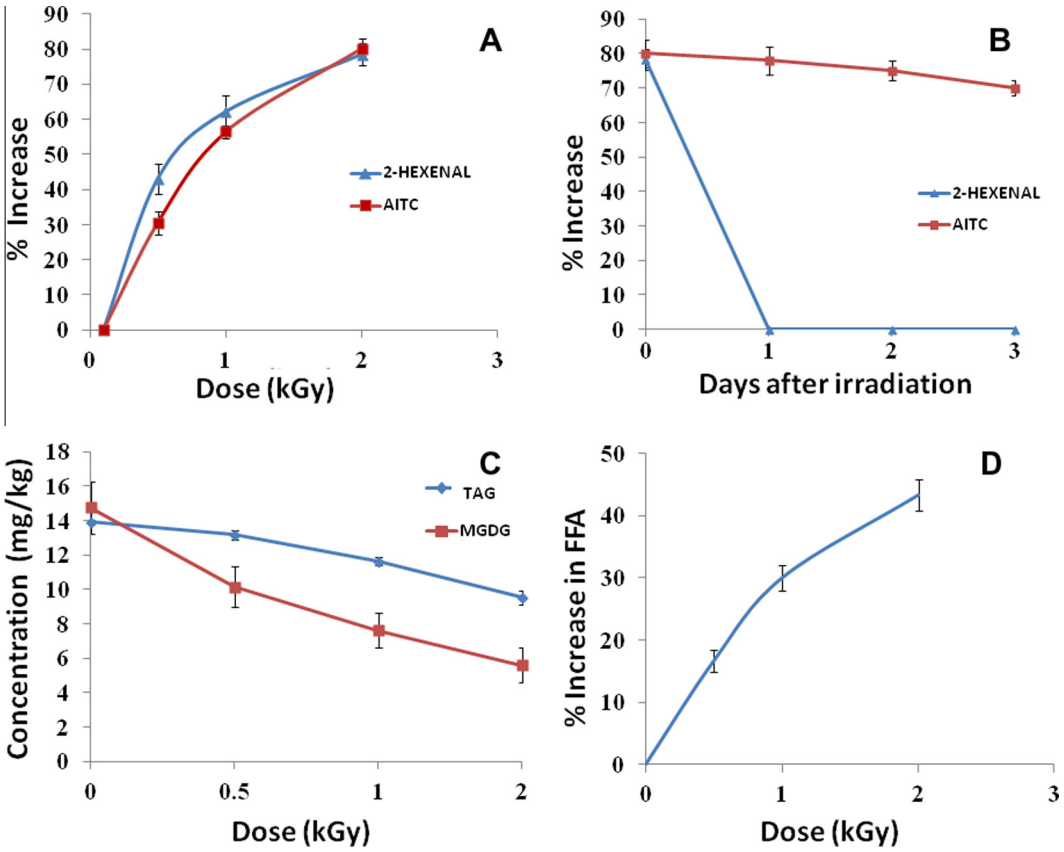

Fig. 1. (A) Effect of radiation dose on AITC and *trans*-hex-2-enal content. (B) Effect of storage after irradiation (2 kGy) on AITC and *trans*-hex-2-enal content. (C) Effect of radiation dose on MGDG and TAG content. (D) Effect of radiation dose on FFA content. Values are expressed as mean ± SD (n = 9).

varieties. Linolenic acid was the major acid in all the varieties followed by linoleic and palmitic acid. The composition of fatty acids is similar to that reported earlier by Peng (1974). The content of linolenic acid has been reported to increase with leaf age. The significant variation in this fatty acid among the different varieties observed here thus reflects their varying maturity. Linolenic acid has been reported as the major fatty acid of MGDG in leaf and stem vegetables (Whitaker, 1986). Hence the nature of the fatty acids in MGDG as well as in TAG, the major lipid species of cabbage, and their contribution to the total fatty acid pool was of interest. Linolenic acid was found to be the major fatty acid in both MGDG and TAG (Table S1). Thus a significant contribution of galactolipids to the linolenic acid content in the total fatty acid profile was inferred.

3.4. Effect of radiation processing on lipid constituents

3.4.1. Effect on lipid species

Effect of radiation processing (2 kGy) on the content of various lipid species identified is shown in Table 2. MGDG was found to be the lipid species most sensitive to radiation processing with a considerable decrease in its content during irradiation. This decrease was found to be variety dependent with the highest reduction in NS-22 (62%) followed by market variety (39%) and BC-79 (31%). Radiation processing was, however, not found to significantly affect the content of DGDG. MGDG has been reported to be the lipid species most sensitive to stress (Matsui, Kurishita, Hisamitsu, & Kajwara, 2000). The content of TAG also showed a considerable decrease albeit lower than MGDG in the irradiated vegetable. Its content decreased by 32.3% in irradiated NS-22 while the corresponding decrease in market variety and BC-79 was 27.8% and 12.6% respectively. A decrease in TAG content as a consequence of radiolysis in irradiated nutmeg was demonstrated by us earlier (Niyas, Variyar, Gholap, & Sharma, 2003). Fig. 1C illustrates the effect of radiation processing at various doses on the content of TAG and MGDG. A linear decrease of TAG from 13.16 to 9.52 mg/kg and MGDG from 10.14 to 5.6 mg/kg was noted when the vegetable was irradiated in the dose range from 0.5 kGy to 2 kGy. The enhanced free fatty acid content observed (Table 2) in irradiated (2 kGy) samples in all varieties and their increase with dose (Fig. 1D) as demonstrated in NS-22 variety further supports the breakdown of the different lipid species such as MGDG and TAG during radiation processing.

3.4.2. Effect on fatty acids

Linoleic and linolenic acids are the major fatty acids demonstrated to be the precursors in the formation of volatile aliphatic C_6 aldehydes and alcohols, also termed as GLVs. In the present study an enhanced break down of MGDG compared to other lipid species and its contribution to the total fatty acid profile suggests the possible role of this galactolipid in contributing to the enhanced *trans*-hex-2-enal content during radiation processing. Galactolipids as preferential substrate of lipase over phospholipids and triglycerides in GLV production has been reported (Matsui et al., 2000). A significant decrease in linolenic acid content was observed in the radiation processed samples of all the three varieties studied (Table 2). A decrease in content of this acid by 41.3%, 34% and 17.6% was noted in NS-22, market sample and BC-79 respectively Linolenic acid is reported to be the precursor of *trans*-hex-2-enals and *cis*-hex-3-enols (Hatanaka, 1996). In the present study the decrease in linolenic acids was found to be linearly correlated (R^2 = 0.99) with the increased *trans*-hex-2-enal

Table 2
Effect of irradiation (2 kGy) on lipid composition in 3 different varieties of cabbage.

Lipid species	NS 22 (mg/kg)		BC-79 (mg/kg)		Market sample (mg/kg)	
	Control	Irradiated	Control	Irradiated	Control	Irradiated
MGDG	14.74 ± 2.11[a]	5.6 ± 0.97[b]	17.12 ± 0.91[a]	11.79 ± 0.41[b]	15.11 ± 0 .31[a]	9.2 ± 0.12[b]
DGDG	3.41 ± 0.11[a]	3.04 ± 0.3[a]	12.98 ± 0.8[a]	13.02 ± 0.04[a]	10.12 ± 0.22[a]	9.98 ± 0.16[a]
TAG	13.98 ± 0.13[a]	9.46 ± 0.39[b]	20.45 ± 1[a]	17.88 ± 0.76[b]	16.99 ± 0.81[a]	12.26 ± 0.64[b]
Fatty acid ester	1.58 ± 0.14[a]	1.97 ± 0.62[a]	3.18 ± 0.09[a]	3.01 ± 0.12[a]	1.98 ± 0.09[a]	2.08 ± 0.19[a]
FFA	5.5 ± 0.11[a]	7.86 ± 0.14[b]	5.8 ± 0.09[a]	6.38 ± 0.05[b]	7.5 ± 0.04[a]	9.3 ± 0.1[b]
Sterol	5.6 ± 0.07[a]	5.1 ± 0.19[a]	6.1 ± 0.44[a]	6.5 ± 0.3[a]	6.6 ± 0.2[a]	6.9 ± 0.15[a]
Phosphatidyl-ethanolamine	1.01 ± 0.07[a]	0.98 ± 0.1[a]	2.31 ± 0.12[a]	1.99 ± 0.39[a]	2.6 ± 0.4[a]	2.1 ± 0.42[a]
Phosphatidyl-inositol	1.1 ± 0.2[a]	1.2 ± 0.3[a]	2.1 ± 0.21[a]	1.98 ± 0.9[a]	2.89 ± 0.3[a]	2.85 ± 0.09[a]
Phosphatidyl-choline	0.98 ± 0.07[a]	1.01 ± 0.05[a]	1.56 ± 0.76[a]	1.69 ± 0.12[a]	2.1 ± 0.07[a]	1.98 ± 0.1[a]
Myristic acid	0.19 ± 0.005[a]	0.22 ± 0.06[a]	0.13 ± 0.007[a]	0.14 ± 0.006[a]	0.29 ± 0.019[a]	0.3 ± 0.002[a]
Pentadecanoic acid	0.10 ± 0.011[a]	0.11 ± 0.004[a]	0.12 ± 0.022[a]	0.13 ± 0.034[a]	0.17 ± 0.018[a]	0.18 ± 0.005[a]
Palmiticic acid	1.9 ± 0.034[a]	1.9 ± 0.036[a]	1.3 ± 0.04[a]	1.3 ± 0.06[a]	2.1 ± 0.023[a]	1.9 ± 0.033[a]
Stearic acid	0.019 ± 0.003[a]	0.019 ± 0.002[a]	0.016 ± 0.002[a]	0.016 ± 0.005[a]	0.018 ± 0.001[a]	0.016 ± 0.011[a]
Oleic acid	0.082 ± 0.004[a]	0.13 ± 0.007[b]	0.062 ± 0.006[a]	0.11 ± 0.002[b]	0.1 ± 0.007[a]	0.18 ± 0.005[b]
Linoleic acid	0.98 ± 0.05[a]	0.99 ± 0.03[a]	1.05 ± 0.02[a]	1.11 ± 0.09[a]	1.56 ± 0.11[a]	1.51 ± 0.18[a]
Linolenic acid	2.9 ± 0.07[a]	1.7 ± 0.04[b]	4.24 ± 0.09[a]	3.49 ± 0.11[b]	3.35 ± 0.07[a]	2.21 ± 0.06[b]
Eicosanoic acid	0.02 ± 0.03[a]	0.021 ± 0.04[a]	0.036 ± 0.06[a]	0.04 ± 0.08[a]	0.026 ± 0.07[a]	0.02 ± 0.06[a]

Data are expressed as mean ± standard deviation (n = 9). Mean values in the same column for a variety bearing different superscripts are significantly different ($p < 0.05$).

content in the irradiated samples (Fig. 2A). Thus an enhanced formation of linolenic acid as a result of radiolysis and a consequent oxidation of the liberated fatty acid to *trans*-hex-2-enal via lipoxygenase pathway could be inferred. It may be noted here that the content of other GLVs such as *cis*-hex-3-enol and *n*-hexanal were unaffected by radiation processing. *cis*-Hex-3-enol is normally formed by reduction of 3-hexenal via alcohol dehydrogenase. However, 3-hexenal was not detected in the volatile oils presently studied. This could explain the absence of any enhancement in *cis*-hex-3-enol as a result of radiation processing. *n*-Hexanal is reported to be formed from linoleic acid via the LOX pathway. No change in linoleic acid content was noted in the irradiated samples (Table 2) in the present study thus explaining the absence of enhanced *n*-hexanal as a result of radiation processing.

3.5. Effect of radiation processing on the enzymes in the LOX pathway

An increased formation of *trans*-hex-2-enal in the vegetable immediately after gamma irradiation suggested the possible activation of the enzymes such as acyl hydrolase, lipoxygenase or HPLs. It was therefore of interest to understand the role of these enzymes in enhancing the content of the aldehyde during processing. Lipase activity of extracts was studied in all the cabbage samples subjected to three different radiation doses (0.5, 1 and 2 kGy). Activities of the enzymes ranged from 0.019 to 0.021 micromol/min/g fresh weight (FW) (Table 3). Lipases, especially galactolipases, are known to be induced by salt and mechanical stress (Matsui et al., 2000). However, in the present study, no significant difference in lipase activity was observed between control and irradiated samples (Table 3). Zhuang, Hamilton-Kemp, Andersen, and Hildebrand (1992) have earlier reported the role of 18:3 rich galactolipids as the possible direct substrate for LOX/HPL without the need for lipases for production C6 aldehyde. Thus the limited role of this enzyme in the production of *trans*-hex-2-enal in the present study could be inferred. The effect of radiation treatment at the above doses on the activities of lipoxygenase and HPL were therefore further examined. Among the stress factors investigated, wounding, jasmonic acid treatment, or pathogen attack are reported to induce LOXs and HPLs (Matsui, 2006). Byun et al. (1996) have earlier reported a negative correlation between the irradiation dose and the lipoxygenase activity. They reported a 71% inhibition of lipoxygenase activity when soybeans were irradiated at 100 kGy. In the present study, LOX activity ranged from 0.88 to 1.05 μmol/min/g FW (Table 3) while HPL activity was found to be between 1.32 and 1.45 μmol/min/g FW (Table 3) at the three doses investigated indicating no significant effect of radiation on the activity of these enzymes. Activities were also determined at different time intervals after irradiation (1 h, 5 h and 1 day). No change in the enzyme activity was observed at all the intervals studied. Thus radiation processing was found to have no impact on the activity of the enzymes of the LOX pathway. Further, addition of crude cabbage extract to linoleic and linolenic acid resulted in the formation of *n*-hexanal and *trans*-hex-2-enal respectively as the end products (Fig. S2), while these compounds were not formed when linoleic and linolenic acid were directly subjected to radiation processing *in vitro*. This confirms the role of the enzymes in the formation of the above C_6 volatiles. In their earlier work on the elucidation of mechanism of GLVs during wounding, Bate and Rothstein (1998) have also observed an enhanced liberation of C_6 volatiles without affecting enzyme activities. They postulated that membrane damage due to wounding caused release of high content of free fatty acids that led to release of C6 volatiles without activation of enzymes of LOX pathway. Increased free fatty acid content is reported to be absolutely essential to meet the demand for C_6 volatiles formation during stress. Lipid radiolysis and consequent enhanced free fatty acid availability was noted in the radiation processed cabbage in the present study. Thus enhanced pool of free linolenic acid consequently formed, results in a greater substrate availability resulting in greater release of *trans*-hex-2-enal without activation of the enzymes of the LOX pathway.

3.6. Estimation of sinigrin content

The above data on volatile constituents clearly demonstrate an enhanced AITC content in the radiation processed cabbage. As this volatile compound is known to be derived from sinigrin, the distribution of this predominant glucosinolate of the vegetable in different varieties was further investigated. Fig. S3 provides a representative HPLC profile of the glucosinolates present in the *n*-butanol extract of NS-22 cabbage variety. On desulfation these peaks were no longer detected confirming them to be glucosinolates (Fig. S3B). The major peak at R_t (11.6 min, Fig. S3A) was identified as sinigrin from its mass spectrum (m/z; 358 M^+) when subjected to LC/MS analysis and by comparison of its R_t with standard injected under similar condition. Table 4 provides the quantitative distribution of sinigrin in different cabbage varieties.

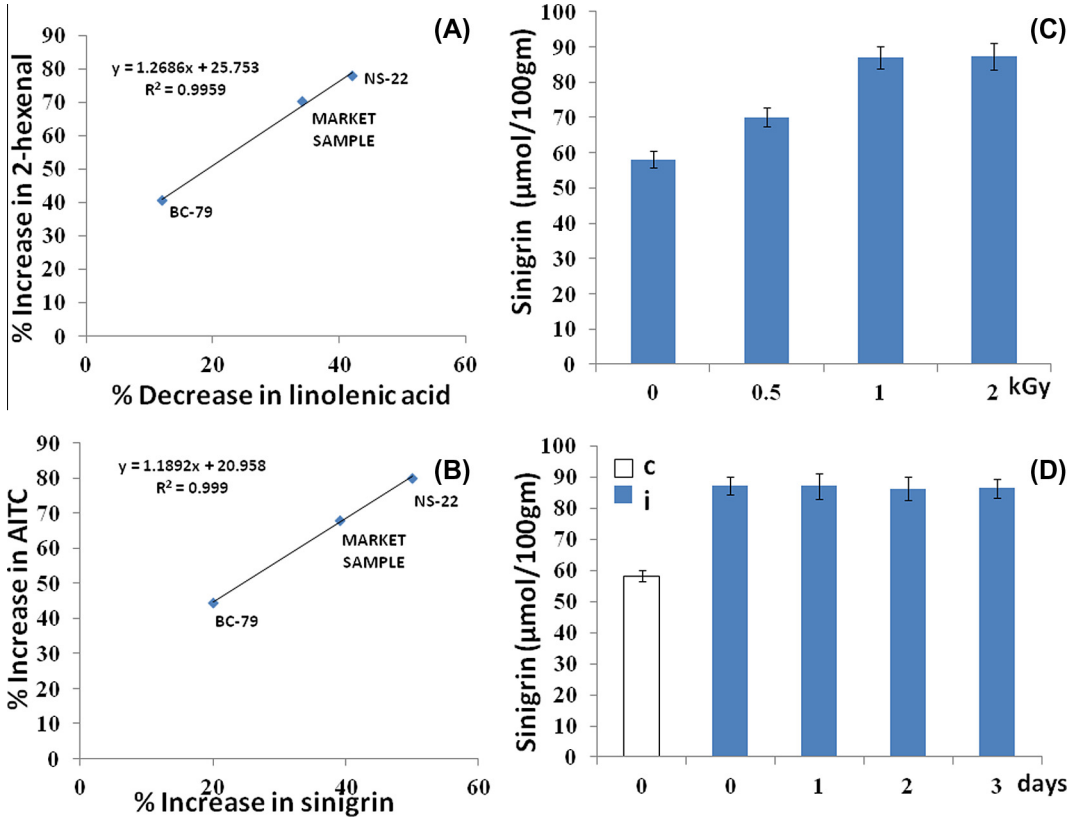

Fig. 2. (A) Plot depicting the relation between *trans*-hex-2-enal and linolenic acid content in irradiated cabbage. (B) Plot depicting the relation between AITC and sinigrin content in irradiated cabbage. (C) Effect of radiation dose on sinigrin content. (D) Effect of storage after irradiation (2 kGy) on sinigrin content. Values are expressed as mean ± SD (n = 9). c-Control, i-Irradiated.

A wide variation in the content of the glucosinolate was noted with the highest in NS-22, followed by BC-79 and market samples. Variation in the content of this glucosinolate among different cabbage accessions ranging from 21.1 to 4.3 µmol g^{-1} DW (dry weight) was reported by Kushad et al. (1999). Song and Thornalley (2007) have reported a sinigrin content of 5.09 ± 1.76 µmol/100 g in fresh green cabbage while its values were found to range from 41.0 to 28.2 µmol/100 g in fresh red cabbage (Dekker & Verkerk, 2003). The observed values of sinigrin content in the present study are in the range reported in literature. Differences in glucosinolate distribution pattern in *Brassica* have been observed between species and ecotype as well as between varieties and even within individual plants, depending on developmental stage, tissue and photoperiod (Martínez-Ballesta, Moreno, & Carvaja, 2013). As glucosinolates account for the distinctive flavours of cabbage, the wide variation observed in the sinigrin content between the different varieties can have a significant impact on their aroma and taste quality.

3.7. Effect of radiation processing on sinigrin content

There is a limited understanding of the effects of post-harvest storage and processing on the glucosinolate content of *Brassica* vegetables. Refrigeration (4–8 °C), freezing, shredding and cooking in boiling water has been reported to significantly decrease glucosinolates in broccoli, brussel sprouts, cauliflower and green cabbage (Song & Thornalley, 2007). No changes in the content of glucosinolates were, however, noted when these vegetables were subjected to steaming, microwave cooking and stir-fry cooking. Oerlemans, Barrett, Suades, Verkerk, and Dekker (2006) have demonstrated a high thermal stability of glucosinolates during blanching (8%) compared to canning (75%) that involves more drastic heat treatment. On the other hand an increase in indole and aliphatic glucosinolates was noted during controlled-atmosphere storage of broccoli for a period of 7 days at 7–13 °C. The effect of post-harvest processing by ionising radiations, a cold process, on the glucosinolate content at doses recommended for microbial decontamination of fresh leafy green *Brassica* vegetables have, however, not been investigated so far. Table 4 shows the effect of radiation processing (2 kGy) on the glucosinolate content. An increase in the content of sinigrin was noted immediately after irradiation in the present study. The highest increase was noted in NS-22 (50%) followed by market samples (39%) and BC-79 (20%). The effect of radiation processing at three different doses of radiation on sinigrin content was further investigated. A linear increase was observed from 0.5 to 1 kGy that remained constant beyond a dose of 1 kGy (Fig. 2C). This increase was noted immediately after irradiation that remained constant on subsequent storage (Fig. 2D). A good correlation was also noted between increase in AITC and sinigrin content (Fig. 2B). Thus the increased AITC observed in the steam distilled volatile oils from radiation processed vegetable could be the result of hydrolytic breakdown of more available sinigrin in the treated samples. A number of environmental conditions such as temperature, light, salinity plant nutritional status, fungal infection, wounding and insect damage can enhance glucosinolate content significantly (Martínez-Ballesta et al., 2013). Mewis et al. (2012) have recently reported an increase in aliphatic glucosinolates in broccoli sprouts on exposure to UV-B radiation. They demonstrated that this increase on exposure to UV-B was a result of up-regulation of genes involved in glucosinolate biosynthesis. A similar effect at genetic level could possibly account for the enhanced sinigrin observed in the present study. This however requires further investigation.

Table 3
Activities (μmol/min/g of fw) of different enzymes of cabbage subjected to different radiation doses.

Enzyme	Control	0.5 kGy	1 kGy	2 kGy
Lipase	0.02 ± 0.009[a]	0.02 ± 0.01[a]	0.0185 ± 0.009[a]	0.021 ± 0.003[a]
LOX	0.995 ± 0.08[b]	0.96 ± 0.0213[b]	0.88 ± 0.09[b]	1.045 ± 0.16[b]
HPL	1.32 ± 0.21[c]	1. 45 ± 0.33[c]	1.44 ± 0.43[c]	1.32 ± 0. 48[c]
Myrosinase	1.286 ± 0.96[d]	1.35 ± 0.43[d]	1.283 ± 0.54[d]	1.31 ± 0.31[d]

Data are expressed as mean ± standard deviation ($n = 9$). Mean values in the same column for a variety bearing different superscripts are significantly different ($p < 0.05$).

Table 4
Effect of irradiation (2 kGy) on sinigrin content in 3 varieties of cabbage.

Variety	Control (μmol/100 g of fw)	Irradiated (μmol/100 g of fw)
NS-22	58.15 ± 2.7[a]	87.23 ± 5.08[b]
BC-79	35.8 ± 2.2[a]	42.96 ± 4.98[b]
Market sample	24.1 ± 2.8[a]	33.5 ± 3.9[b]

Data are expressed as mean ± standard deviation ($n = 9$). Mean values in the same column for a variety bearing different superscripts are significantly different ($p < 0.05$).

3.8. Effect of radiation processing on myrosinase activity

Tissue damage as result of postharvest processing of the vegetable can result in cellular breakdown and a consequent hydrolysis of glucosinolates by endogenous myrosinase. The presence of active myrosinase is a prerequisite for formation of bioactive breakdown products of glucosinolates such as isothiocyanates that in turn determine their final intake levels. There are however very few reports on the effect of postharvest processing on myrosinase activity. Earlier work has reported a loss of myrosinase activity in cabbage after 2 min of microwave cooking and after 7 min of steaming (Rungapamestry, Duncan, Fuller, & Ratcliffe, 2006). Dekker and Verkerk (2003) have also demonstrated a diminished myrosinase activity with increasing input of microwave energy. In the present study the enhanced AITC observed in the essential oils of irradiated vegetable suggests either an increased myrosinase activity or a greater enzyme substrate interaction as a consequence of increased sinigrin availability. No significant change in the myrosinase activity was observed as a result of radiation processing at doses of 0.5–2 kGy in the present study (Table 3). In an earlier work, Lessman and McCaslin (1987) have reported inactivation of myrosinase without degradation of glucosinolates when mustard and rape were exposed to gamma radiation dose of 5 kGy. Lower doses presently employed may possibly account for the ineffectiveness of radiation treatment in affecting myrosinase activity. *In vitro* exposure of sinigrin in aqueous solution to radiation processing further ruled out the formation of volatile AITC by direct radiolysis of sinigrin (data not shown). Thus retention of myrosinase activity can aid in greater availability of bioactive degradation products from the increased sinigrin formed as a consequence of radiation processing and thus enhance their final intake levels.

4. Conclusion

Modulating glucosinolate profile has been one of the current strategies to enhance health promoting properties and thus improve the nutraceutical value of Brassica vegetables. Conventional food processing methods including cooking have been found to reduce both the glucosinolate content and myrosinase activity. This has resulted in a lower release of protective breakdown products thus reducing their intake. Post harvest processing methods that provide high retention of glucosinolates can facilitate improved release of health promoting compounds during mastication of these vegetables. The current work has demonstrated the feasibility of radiation processing as an effective post harvest processing method in enhancing glucosinolate content while retaining myrosinase activity. Irradiation is known to control insect infestation, reduce pathogenic bacteria and delay natural processes like ripening, germination or sprouting in fresh food. Thus besides being highly effective method of ensuring food safety and extending shelf life the method provides improved benefit in terms of enhancing intake of potentially important health protective and promoting compounds and flavour quality.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2013.11.055.

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Short communication

Gamma irradiation inhibits wound induced browning in shredded cabbage

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ABSTRACT

Gamma-radiation induced browning inhibition in minimally processed shredded cabbage stored (10 °C) for up to 8 days was investigated. γ-irradiation (2 kGy) resulted in inhibition of browning as a result of down-regulation (1.4-fold) in phenylalanine ammonia lyase (PAL) gene expression and a consequent decrease in phenylalanine ammonia lyase (PAL) activity. Activity of polyphenol oxidase and peroxidase, total and individual phenolic content as well as *o*-quinone concentration were, however, unaffected. In the non-irradiated samples, PAL activity increased as a consequence of up-regulation of PAL gene expression after 24 and 48 h by 1.2 and 7.7-fold, respectively, during storage that could be linearly correlated with enhanced quinone formation and browning. Browning inhibition in radiation processed shredded cabbage as a result of inhibition of PAL activity was thus clearly demonstrated. The present work provides an insight for the first time on the mechanism of browning inhibition at both biochemical and genetic level.

1. Introduction

Amongst the physiological factors limiting post harvest storage of fresh plant produce, enzymatic browning plays a major role in reducing sensory quality and nutritional value of these products (He & Luo, 2007). Enzymatic browning thus causes significant economic losses to the fresh produce industry. Browning is associated with the loss of cellular integrity and de-compartmentalisation in response to cellular injury (He & Luo, 2007). It mainly involves metabolism of phenolic compounds. In intact plant cells, phenolic compounds in cell vacuoles are spatially apart from the oxidising enzymes present in the cytoplasm. Once tissues are damaged by cutting, grinding or pulping, the mixing of the enzymes and phenolic compounds as well as the easy oxygen diffusion to the inner tissues result in a browning reaction.

In response to tissue injury, phenylalanine ammonia lyase (PAL) produces phenols which are then oxidised by polyphenol oxidase (PPO) and peroxidase (POD) to *o*-quinones that further polymerise to brown pigments (He & Luo, 2007). A basic understanding of the processes leading to browning is needed for developing better approaches in enhancing the post harvest shelf life of fresh produce.

A number of chemical additives like ascorbic acid and citric acid are used as browning inhibitors for fresh produce (He & Luo, 2007). In addition, sanitizers, such as ozone and chlorine, are commonly used for controlling microbial load in fresh-cut products (He & Luo, 2007). Being oxidising in nature, sanitizers work antagonistically to browning inhibitors, which are usually reducing in nature. Consequently, in combination, they usually cancel out each other's desired effects. Thus, at present a single treatment that can effectively prolong the shelf-life of fresh-cut products, while preventing browning and maintaining product quality and safety for consumers has limited availability (He & Luo, 2007). Hence, a disinfectant that can work in conjunction with anti-browning reagent or which itself can act as a browning inhibitor could have widespread application in the food industry. In our recent work on shelf life extension of shredded cabbage using γ-irradiation we found an effective inhibition of cut edge browning (unpublished). Use of γ-irradiation for eliminating pathogenic and spoilage microorganisms to ensure safety of fresh fruits and vegetables has been widely reported (Arvanitoyannis, Stratakos, & Tsarouhas, 2009). However, use of γ-irradiation for browning inhibition in fresh-cut produce has not been investigated extensively.

The present work focuses on understanding the mechanism of browning during wounding in shredded cabbage and its inhibition by γ-irradiation. To the best of our knowledge this is the first report on the mechanism of inhibition of browning by γ-irradiation in any fresh-cut vegetable.

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0308-8146/

2. Materials and methods

2.1. Materials

Cabbage (*Brassica oleracea*, BC-79 variety) samples were obtained from Dr. Panjabrao Deshmukh Krishi Vidyapeeth, India. Harvesting was carried out 65 days after planting when the above variety is known to be mature.

2.2. Irradiation and storage

Cabbage samples were washed with tap water and healthy, fresh samples were selected. Samples (40 g) were cut into 1 cm wide by 3.0–3.5 cm long strips and packed into polystyrene trays (9 cm × 9 cm × 2.5 cm). The trays were over-wrapped all around with cling film (Flexo film wraps Ltd., Aurangabad, India).

Packaged samples were subjected to different radiation doses (0.5, 1.0 and 2.0 kGy) in a cobalt-60 irradiator (GC-5000, BRIT, Mumbai, India) at a dose rate of 3.34 kGy/h. Samples were stored in the dark at 10 ± 1 °C.

2.3. Evaluation of browning

Browning of the cut edges were measured by Minolta Chromameter (model CM-3600d Konica Minolta Sensing Inc., Japan) as described previously (Tripathi, Chatterjee, Vaishnav, Variyar, & Sharma, 2013).

Tissues were also scored visually. The rating scale reported by Ke and Saltveit (1986) was used to visually estimate the extent of browning in cut edges: with 0 (no browning) and 9 (complete browning).

2.4. Total phenolic content

Forty grams of cabbage were extracted twice in 150 ml of aqueous methanol. The extract was centrifuged and the supernatant concentrated to make 1% solution. Total phenolic content (TPC) was evaluated in accordance with the Folin–Ciocalteu procedure (Singleton & Rossi, 1968). TPC was expressed as mg GAE (Gallic acid equivalent) 100 g^{-1} fresh weight (FW) of cabbage.

2.5. HPLC analysis of phenolic compounds

The methanol extract was subjected to HPLC (Jasco HPLC system, Japan) using 0.1% formic acid (solvent A) and methanol (solvent B) using an RP C-18 (HYPERSIL, Chromato-pack, India) column (250 mm × 4.6 mm, 10 μ) and solvent gradient: time 0 min, A = 80%; time 35 min, A = 50%; time 37 min, A = 20%; flow rate: 1 ml/min; wavelength: 280 nm (Ferreres et al., 2005). The phenolics were identified by comparing their Rt (retention time) with standard compounds. Co chromatography with added standards was also performed for further confirmation of the identified compounds. Peak quantification was achieved by use of calibration curves obtained for each reference standard.

2.6. Enzyme assay

PAL, PPO, chlorogenic acid peroxidase (A_{410}) and caffeic acid peroxidase (A_{470}) activities were measured according to Degl'innocenti, Guidi, Pardossi, and Tognoni (2005). One unit of PAL activity equals the amount of PAL that produced 1 μmol of *trans*-cinnamic acid in 1 h and is expressed as μmol g^{-1} FW h^{-1}. One unit of PPO activity was defined as the amount of enzyme that caused an increase in absorbance of 0.01 unit per minute. The activities of PODs are expressed as ΔA_{λ} min^{-1} g^{-1} fresh weight.

2.7. o-Quinone content

Soluble *o*-quinones were extracted as described by Ke and Saltveit (1986).

2.8. RNA extraction and cDNA preparation

RNA extraction was done using TRI reagent (Sigma, T 9424) as per the manufacturer's instructions. The quantity of RNA was measured using a NanoDrop 3300 spectrophotometer (Thermo Scientific, MA) and the integrity was checked by electrophoresis of total RNA (1 μg) on a 1.2% denaturing agarose gel.

One μg of the total RNA was reverse transcribed with Superscript™ III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen, USA) as per manufacturer's instructions.

2.9. Quantitative real time-PCR

Previously reported primer sets of PAL (*PAL1*) and actin (*ACTIN2*) as reference gene for normalisation and quantification were used for qRT-PCR (Table 1) (Srivastava, Ramaswamy, Suprasanna, & D'Souza, 2010). qRT-PCR was carried out using a Corbett rotor gene 3000 (Corbett Life Science, www.corbettlifescience.com). Detection of real-time RT-PCR products was done using a SyBr Green Master Mix kit (S 4320, Sigma), as per the manufacturer's instructions. The PCR cycling conditions comprised an initial cycle at 50 °C for 2 min followed by one cycle at 95 °C for 10 min and 40 cycles each comprising 95 °C for 30 s, 55 °C for 45 s, and 72 °C for 30 s. For each sample, reactions were set up in triplicate to ensure the reproducibility of the results.

At the end of each PCR run, a melting curve was generated and analysed with the dissociation curve software built into the Corbett rotor gene 3000. A relative expression ratio plot was generated using the software REST-MCS.

2.10. ASA and DHA content

Total ascorbic acid (ASA) content was estimated in accordance with the standard official microfluorometric method of AOAC (1990). ASA content was calculated by subtracting the dehydroascorbic acid (DHA) content from total ASA content.

2.11. Statistical analysis

DSAASTAT ver. 1.101 by Andrea Onofri was used for statistical analysis of data. Data was analysed by analysis of variance (ANOVA) and multiple comparisons of means were carried out

Table 1
List of primers used for qRT-PCR.

Target gene	Description	Forward primer (5′–3′)	Reverse primer (5′–3′)
PAL1	*Brassica juncea* phenylalanine ammonia lyase (PAL)	GCCGGAGTATCG	AGCCGCTCTGATC
ACTIN2	*Brassica oleracea* Actin	TCCAGGAATCGTTCACAG	GCTACAAAACAATGGGAC

using Duncan's multiple range test. Data are expressed as means ± SD of three independent analyses each carried out in triplicate. Means are expressed as significantly different or not at 5% level of confidence.

3. Results and discussion

Cabbage, an important member of the Brassica family, is known for its nutritional value. It is widely marketed as a minimally processed product in the shredded form due to the associated convenience. However, a major limitation in its post harvest storage is the appearance of browning at the cut edges. To the best of our knowledge no studies exist so far on browning in shredded cabbage. A good understanding of the process can aid in preventing browning and thus enhance the shelf life of the product.

3.1. Enzymatic browning in non-treated control shredded cabbage

3.1.1. Evaluation of browning

Fig. 1 shows the effect of irradiation and storage on cut edge browning of cabbage. The visually evaluated score (Fig. 1B) and the *L* value (Fig. 1A) measured by colorimeter demonstrated similar results wherein an increase in browning intensity was apparent with storage in control samples. Significant browning at the cut edges was seen beyond 4 days of storage which further increased at the end of 1 week. Cut edge browning on storage has been reported previously in a number of vegetables, thus reducing the shelf life of the products (Ke & Saltveit, 1986).

3.1.2. Enzyme assays

Alteration in phenolic metabolism is generally known to affect browning in cut vegetables. PAL is the first enzyme in the phenylpropanoid pathway involved in synthesis of phenolic compounds. In the present study a low PAL activity was observed in the freshly cut cabbage strips. With storage, the activity was found to increase, reaching maxima on day 2 and then remaining constant up to day 4, after which a slight decrease was noted on further storage (Fig. 1C). Several studies on cut lettuce have shown a wound induced enhancement in PAL activity on storage. Degl'Innoceti et al. (2005) for instance, noted a significant increase in PAL activity within 5 h, whereas Hisaminato, Murata, and Homma (2001) found maximum increase after 3 days of storage. Murata, Tanaka, Minoura, and Homma (2004) also found a significant increase in the activity of this enzyme after 3 days of storage that further increased on storage up to day 6. Thus, the effect of wounding on PAL activity was found to vary with the variety of lettuce. Stress induced enhancement in PAL activity has been extensively reported in different plant tissues. Various stresses, such as nutrient deficiencies or viral, fungi and insect attack, are known to increase either PAL synthesis or activity in different plants (He & Luo, 2007). In the present case, shredding of cabbage induced a stress which resulted in an increase in PAL activity. Wound induced enhancement in PAL activity has also been previously reported in minimally processed potatoes (Vitti, Sasaki, Miguel, Kluge, & Morett, 2011). A linear correlation (R^2 = 0.97) between browning and PAL activity (Fig. 2C) further indicated its role in browning.

PPO is a downstream enzyme in the phenylpropanoid pathway acting on phenols to form *o*-quinone. PPO activity of the enzymes ranged from 10.1 to 12.4 U/g fresh weight (FW). PPO activity remained nearly constant during storage in the present study (Table 2). This indicated that PPO activity was high enough in shredded cabbage to cause browning. Other authors have also reported no significant changes in PPO activity in iceberg lettuce

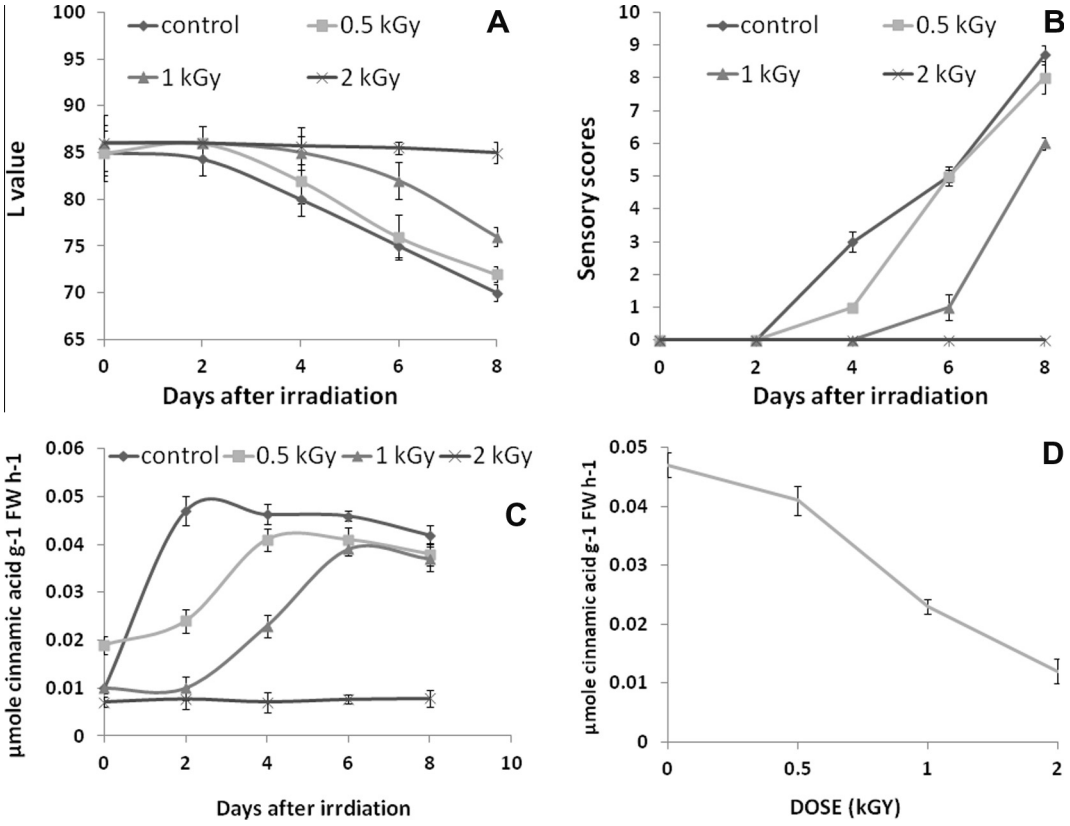

Fig. 1. (A) Effect of radiation treatment on *L* values (B) Effect of radiation treatment on sensory score for browning. (C) Effect of irradiation and storage (8 days) on PAL activity. (D) PAL activities at different doses on day 4. Values are expressed as mean ± SD (n = 9).

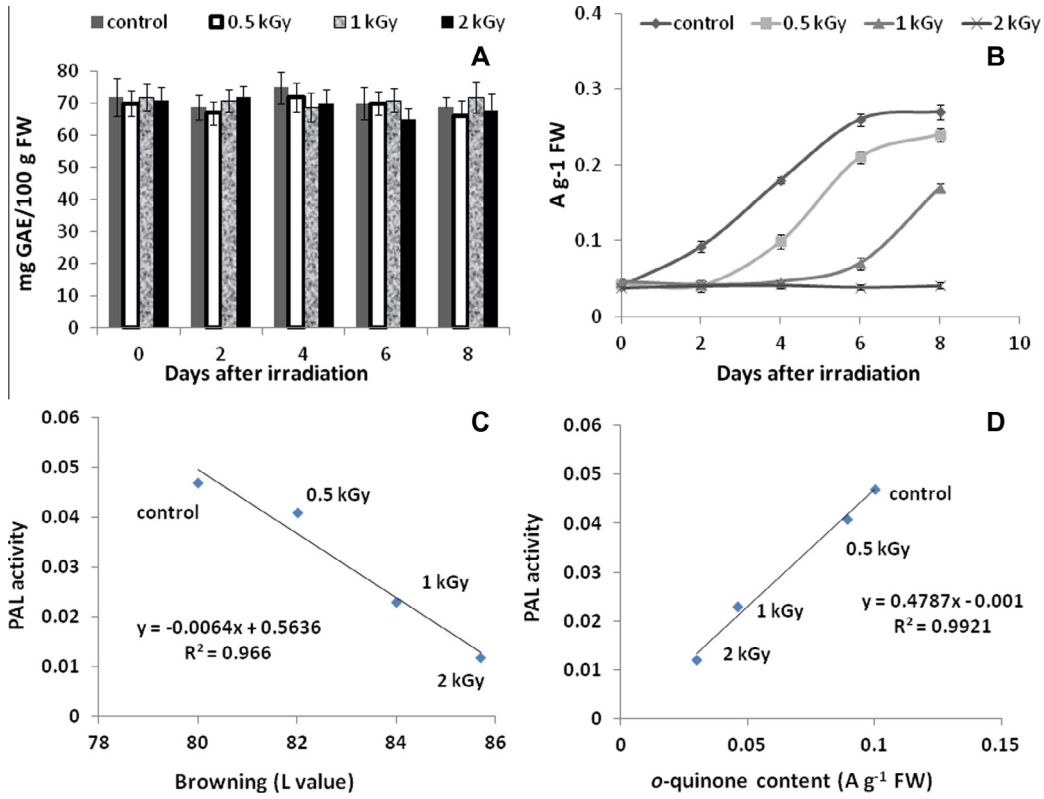

Fig. 2. (A) Effect of irradiation and storage on total phenolic content. (B) Effect of irradiation and storage on soluble *o*-quinone content in shredded cabbage. (C) Plot depicting the relation between PAL activity and browning in shredded cabbage. (D) Plot depicting the relation between PAL activity and *o*-quinone content in shredded cabbage. PAL activity, *o*-quinone content and *L* values were evaluated on 4th day of storage. Values are expressed as mean ± SD ($n = 9$).

Table 2
Effect of irradiation (0.5–2 kGy) and storage on PPO and POD activity of cabbage.

DAY	Control			0.5 kGy			1 kGy			2 kGy		
	PPO	POD1	POD2	PPO	POD1	POD2	PPO	POD1	POD2	PPO	POD1	POD2
0	10.1 ± 2.1^a	6.1 ± 1.1^a	5.4 ± 2.2^a	11.1 ± 2.2^a	6.9 ± 1.9^a	4.9 ± 1.8^a	12.1 ± 4.1^a	7.1 ± 2.4^a	5.1 ± 1.1^a	10.1 ± 3.9^a	6.9 ± 1.9^a	4.6 ± 1.1^a
2	11.1 ± 4.7^a	7.2 ± 2.2^a	6.1 ± 1.8^a	10.7 ± 3.1^a	6.7 ± 1.7^a	5.3 ± 1.2^a	12.3 ± 1.1^a	6.8 ± 1.8^a	5.2 ± 1.2^a	11.1 ± 2.1^a	5.9 ± 2.1^a	5.1 ± 2.1^a
4	12.4 ± 3.1^a	6.3 ± 1.9^a	5.1 ± 1.6^a	12.1 ± 4.2^a	5.8 ± 2.1^a	5.9 ± 2.2^a	10.5 ± 2.8^a	5.9 ± 1.9^a	4.9 ± 2.1^a	12.1 ± 3.3^a	6.1 ± 2.3^a	4.9 ± 1.3^a
6	11.1 ± 2.1^a	5.8 ± 2.1^a	4.1 ± 1.4^a	10.7 ± 2.1^a	6.1 ± 2.2^a	6.1 ± 2.3^a	11.1 ± 4.7^a	7.1 ± 2.1^a	5.2 ± 0.7^a	10.5 ± 3.8^a	6.7 ± 1.9^a	6.1 ± 1.5^a
8	10.2 ± 3.9^a	6.4 ± 2.3^a	4.8 ± 2.1^a	10.9 ± 3.1^a	6.4 ± 1.2^a	4.9 ± 1.7^a	10.9 ± 2.3^a	6.3 ± 2.2^a	4.9 ± 1.1^a	11.1 ± 4.1^a	5.8 ± 2.1^a	4.9 ± 1.3^a

Data are expressed as mean ± standard deviation ($n = 9$). Mean values in the same column bearing same superscript shows no significant difference ($p \leqslant 0.05$). PPO activity is represented in U g^{-1} FW, POD activity is represented in Δ A min^{-1} g^{-1} FW; POD1 is caffeic acid peroxidase activity and POD2 is chlorogenic acid peroxidase activity.

leaf cuts during cold storage (Degl'Innocenti et al., 2005; Hisaminato et al., 2001).

POD is another enzyme almost ubiquitously present in plant, that in the presence of hydrogen peroxide converts a number of phenolics to form *o*-quinone. However, its role in enzymatic browning remains questionable mainly because of the low H_2O_2 content in vegetable tissues (He & Luo, 2007). Free radicals including H_2O_2 are generated due to water radiolysis on irradiation. Consequently, analysis of POD activity is of significance in the present study. POD activity was assayed in the presence of natural hydrogen donors (caffeic and chlorogenic acid). POD activities did not vary substantially during storage for both the substrates (Table 2), thus ruling out its role in browning in shredded cabbage.

3.1.3. Real-time PCR analysis of PAL gene

Shredding and storage in cabbage samples resulted in a change in PAL activity in cabbage samples while no change was noted in the activities of other enzymes. Transcriptional analysis of PAL gene at different storage points was therefore studied. Gene expression was analysed at 0, 24 and 48 h of storage. The expression level of these transcripts at various storage periods was recorded. A comparison of the expression levels of the control sample at 0 h (Fig. 3A) with those at 24 and 48 h showed a gradual increase in PAL gene expression with storage. An up-regulation of 1.2-fold and 7.7-fold was seen after 24 and 48 h, respectively, thus justifying the increase in PAL activity during storage. Similar results were seen in cut lettuce where a 3.4-fold increase was seen in PAL mRNA within 24 h of wounding, which resulted in an increase in PAL activity (Campos-Vargas, Nonogaki, Suslow & Saltveit, 2005).

3.1.4. Phenolic content

PAL catalyses the biosynthesis of phenolic compounds that are subsequently oxidised to brown pigments by PPO/POD. Table 3 lists the major phenolic compounds identified in shredded cabbage. Gallic acid was found to be the major phenolic acid followed by γ-resorcylic acid and chlorogenic acid. Ferulic acid, sinapic acid and ellagic acid were detected in minor amounts. Amongst these,

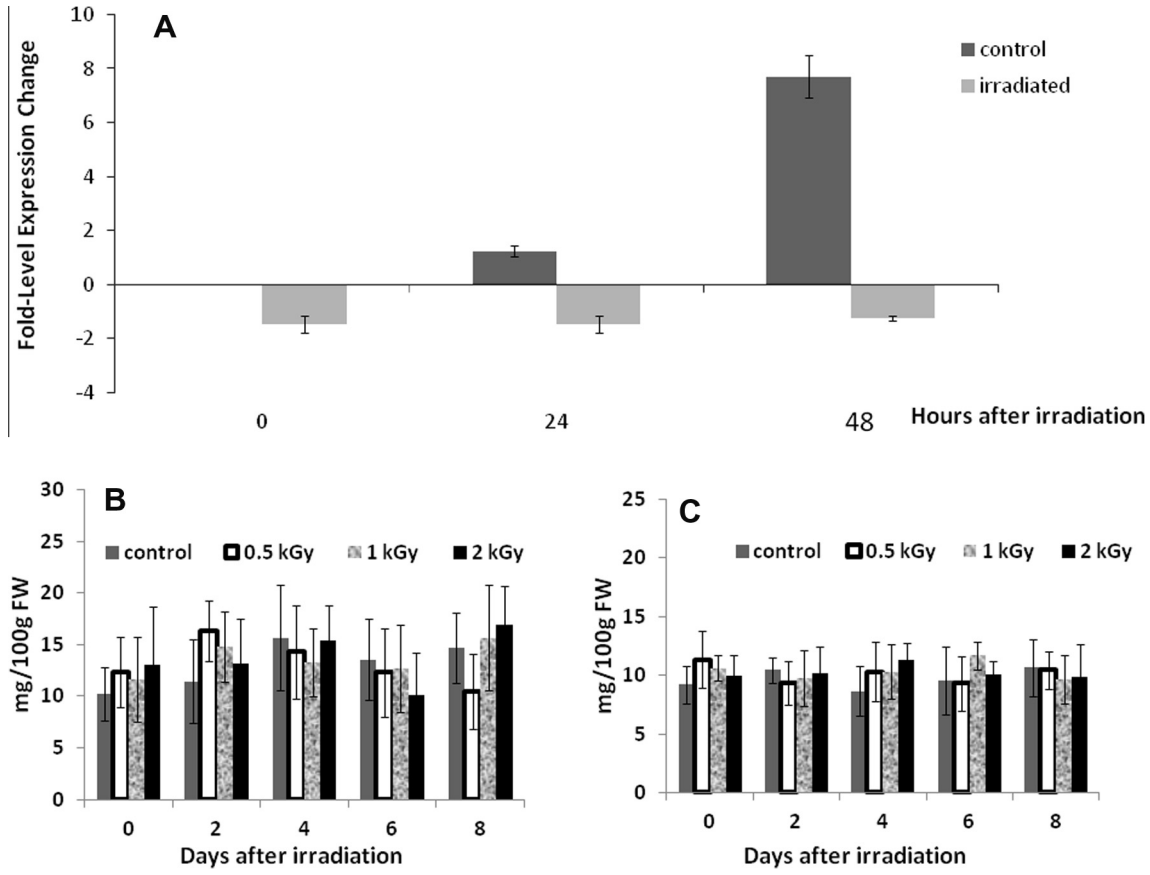

Fig. 3. (A) Fold change in the expression of PAL gene. The *x*-axis represents the expression level of PAL gene in control at 0 h. All values are means of triplicates ± SD. (Irradiation dose = 2 kGy). B) Effect of irradiation and storage on ascorbic acid and C) Effect of irradiation and storage on dehydroascorbic acid content in shredded cabbage. Values are expressed as mean ± SD ($n = 9$).

Table 3
Effect of irradiation (2 kGy) and storage on individual phenolic acid content (mg/kg) of cabbage.

Phenolic Acid	Day 0		Day 2		Day 4		Day 6		Day 8	
	Control	Irradiated	Control	Irradiated	Control	Irradiated	Control	Irradiated	Control	Irradiated
Gallic acid	28.1 ± 4.5^{a}	29.3 ± 5.8 ^{a}	29.1 ± 4.5^{a}	28.3 ± 3.5 ^{a}	29.6 ± 4.5^{a}	28.1 ± 6.6^{a}	28.1 ± 4.5^{a}	27.3 ± 4.9^{a}	29.4 ± 2.3^{a}	29.5 ± 3.1^{a}
γ-Resorcylic acid	6.3 ± 1.1^{b}	6.1 ± 2.1^{b}	5.9 ± 1.2^{b}	6.2 ± 1.4^{b}	5.9 ± 1.1^{b}	5.7 ± 1.5^{b}	6.2 ± 1.5^{b}	5.7 ± 0.9^{b}	5.8 ± 1.6^{b}	6.2 ± 1.2^{b}
Chlorogenic acid	4.2 ± 1.2^{c}	3.8 ± 0.8^{c}	3.8 ± 1.1^{c}	4.1 ± 1.1^{c}	4 ± 1.3^{c}	3.7 ± 1.7^{c}	4.2 ± 1.2^{c}	3.8 ± 1.1^{c}	4.1 ± 0.9^{c}	4.4 ± 1.2^{c}
Ferulic acid	1.5 ± 0.4^{d}	1.1 ± 0.8^{d}	1.8 ± 0.8^{d}	1.3 ± 0.5^{d}	1.3 ± 0.6^{d}	1.7 ± 0.8^{d}	1.2 ± 0.4^{d}	1.3 ± 0.9^{d}	1.2 ± 0.9^{d}	1.5 ± 0.7^{d}
Sinapic acid	1.2 ± 0.2^{e}	1.4 ± 0.4^{e}	1.1 ± 0.3^{e}	1.2 ± 0.6^{e}	1.6 ± 0.5^{e}	1.4 ± 0.5^{e}	1.2 ± 0.2^{e}	1.1 ± 0.5^{e}	1.5 ± 0.2^{e}	1.3 ± 0.2^{e}
Ellagic acid	1.5 ± 0.4^{f}	1.3 ± 0.4^{f}	1.2 ± 0.4^{f}	1.1 ± 0.5 ^{f}	1.4 ± 0.6^{f}	1.6 ± 0.4^{f}	1.1 ± 0.2^{f}	1.1 ± 0.3^{f}	1.2 ± 0.6^{f}	1.1 ± 0.4^{f}

Data are expressed as mean ± standard deviation ($n = 9$). Mean values in the same row bearing same superscript shows no significant difference ($p \leqslant 0.05$).

chlorogenic acid and sinapic acid have been identified in different cabbage varieties (Martínez, Olmos, Carballo, & Franco, 2010; Ferreres et al., 2005). Ferulic acid has been demonstrated to exist in cabbage as quercetin and kaempferol derivative (Cartea, Francisco, Soengas, & Velasco, 2011). Gallic acid, γ-resorcylic acid and ellagic acid have, however, not been previously reported in cabbage. The total phenolic content was found to be comparable to that reported earlier for cabbage (Jaiswal, Rajauria, Abu-Ghannam, & Gupta, 2011). Interestingly, no change in the total or individual phenolic content was observed immediately after processing or on subsequent storage. Degl'Innocenti, Pardossi, Tognoni, and Guidi (2007) also found similar results in cut lettuce and escarole wherein no change in phenolic compounds was noted despite an increase in PAL activity. Rapid oxidation of phenolics was proposed by these researchers. Several studies have shown that accumulation of phenolic compounds in plant cell is not a mere function of the rate of phenolic synthesis but varies strongly in relation to its physiological state and is a result of equilibrium between biosynthesis and further metabolism including turnover and catabolism (Oufedjikh, Mahrouz, Amiot, & Lacroix, 2000). In the present study, we did not find any correlation between phenolic content and browning. Thus equilibrium between phenolic biosynthesis and its further metabolism could possibly explain the non-alteration in total phenolic content. Our results are in accordance with the reports of Hisaminato et al. (2001) and Vitti et al. (2011) for cut lettuce and potato respectively in which no correlation could be established between phenolic content and browning.

3.1.5. *o*-Quinone content

Phenols are converted to *o*-quinones, which in due course either polymerise and/or combine together with amino compounds to form brown pigments. A gradual increase in *o*-quinone content was found in control samples with storage (Fig. 2B). As *o*-quinones

are the oxidised product of phenolic compounds, an equilibrium between phenolic compounds synthesised due to enhanced PAL activity and their corresponding metabolites including quinones could be inferred, thus explaining the absence of any change in phenolic content. Further, quinone content also showed good correlation ($R^2 = 0.99$) with PAL activity (Fig. 2D) as well as with browning, thereby establishing a direct relationship between increase in PAL activity and browning.

3.2. Effect of γ-irradiation on cut edge browning in shredded cabbage

3.2.1. Evaluation of browning

γ-Irradiation induced browning inhibition has been reported in earlier studies of cut vegetables. However, no studies have dealt on the mechanism of browning inhibition in these products. In the present study, γ-irradiation was found to have an inhibitory effect on browning in shredded cabbage. With an increase in irradiation dose the extent of browning was found to decrease, with complete browning inhibition at 2 kGy for 8 days. In the 0.5 kGy sample, browning of cut edges was seen beyond 6 days of storage, whereas, in the 1 kGy sample browning could be observed only at the end of the storage period (8 days). In samples exposed to a dose of 2 kGy no visual browning was seen throughout the storage period. A similar observation has been made by Ke and Saltveit (1986) in various fresh-cut vegetables wherein a gamma radiation dose dependent inhibitory effect on browning was noted. A similar finding has been made by Tripathi et al. (2013), whereby a dose of 2 kGy was found to be effective in inhibiting cut edge browning in ash gourd cubes. On the other hand, irradiation induced browning has been reported in potato tubers, mushrooms, tropical fruits and in cut witloof chicory wherein a dose of 3 kGy was found to induce browning during storage. Tanaka and Langerak (1975) described the browning process to be non-enzymatic, arising due to the generation of free radicals on irradiation. Hanotel, Fleuriet, and Boisseau (1995) on the other hand, found an increase in PAL activity during gamma irradiation to be responsible for the enhanced browning observed. In view of the contradictory observations, a detailed study on the browning process is envisaged for a better understanding of the browning inhibition during radiation processing as currently observed.

3.2.2. Enzyme assays

The effect of γ-irradiation and storage on PAL activity of shredded cabbage is shown in Fig. 1C. The 0.5 kGy sample showed a small increase in enzyme activity immediately after irradiation, which increased gradually with storage reaching a maximum value on day 4. Induction of PAL activity at a low dose of γ-irradiation has been previously reported by Pendharkar and Nair (1975). In the 1 kGy treated sample no significant change ($p \leqslant 0.05$) in PAL activity was seen immediately after irradiation. However, on storage a small increase was noted beyond day 4 that reached maxima at the end of storage period. The sample exposed to a dose of 2 kGy showed a small increase ($p \leqslant 0.05$) in PAL activity immediately after irradiation which remained constant on further storage. Since significant browning was seen in control samples from day 4 onwards, the enzyme activity on this day, in samples treated with different doses, were compared (Fig. 1D). A dose dependent decrease was seen in PAL activity, thus confirming the role of γ-irradiation in inhibiting PAL activity. Benoit, D'Aprano, and Lacroix (2000) also found a decrease in PAL activity in mushrooms, resulting in retention of whiteness of the sample. Our results are in contrast with the earlier reports on cut witloof chicory and potato tubers, wherein an increase in PAL activity due to γ-irradiation has been reported (Hanotel et al., 1995; Pendharkar & Nair, 1975). No effect of gamma irradiation on PPO and POD activities were observed.

3.2.3. Real-time PCR analysis of PAL gene

Since a dose of 2 kGy was found to be effective in inhibiting browning in shredded cabbage throughout storage, transcriptional analysis of PAL gene was performed for this dose. Gene expression of irradiated samples was analysed at 0, 24 and 48 h after irradiation. The expression level of these transcripts was recorded in comparison with their expression in control sample at 0 h (Fig. 3A). Immediately after irradiation, down regulation in PAL gene expression was observed by 1.4-fold that clearly accounted for the decrease in PAL activity. The gene expression further remained constant with storage, thereby explaining the constant enzyme activity in 2 kGy samples on subsequent storage. The effect of γ-irradiation on PAL gene expression has not been previously reported. However, heat shock induced thermal degradation of PAL mRNA has been previously reported in yeast (Lindquist, 1981). Heat shock induced repression of PAL activity resulting in browning inhibition has been reported by Murata et al. (2004) in cold stored cut lettuce. Consequently, post harvest stress type treatments, such as γ-irradiation and heat shock, may act in a similar mode on phenolic metabolism pathways, resulting in browning inhibition.

3.2.4. Phenolic and o-quinone content

Total and individual phenolic content remained unaffected during irradiation and subsequent storage. This could be explained by the constant PAL activity in the irradiated samples. Similar to PAL activity, *o*-quinone content also decreased with increase in irradiation dose (Fig. 2B). In samples exposed to a dose of 0.5 kGy *o*-quinone content was found to increase from day 4 onwards, while in 1 kGy sample this increase was noted on the 8th day. No change ($p \leqslant 0.05$) in soluble *o*-quinone content was found throughout the storage period in samples given a dose of 2 kGy. The quinone content showed good correlation ($R^2 = 0.99$) with PAL activity (Fig. 2D) as well as with browning, thus further establishing a direct relationship between increase in PAL activity and browning.

3.3. Effect of γ-irradiation on non-enzymatic browning in shredded cabbage

Non-enzymatic browning in vegetables, although of less significance, can also occur during storage. ASA present in appreciable amounts in vegetables is known to be converted non-enzymatically to DHA on storage that can degrade into brown pigments. Degl'innocenti et al. (2005) have reported a liner correlation between conversion of ASA to DHA and the occurrence of browning in fresh-cut lettuce leaves. Therefore, the content of ASA and DHA in control and irradiated samples during storage was investigated (Fig. 3B and C). ASA content estimated in the present study (10.11–16.9 mg/100 g) is in agreement with the previous reports available (Singh, Upadhyay, Prasad, Bahadur, & Rai, 2007). DHA content was found to be slightly lower, ranging from 8.67 to 11.67 mg/100 g. The content of ASA and DHA in control and irradiated sample remained constant throughout the storage period of 8 days, thus ruling out the possibility of non enzymatic browning in the present case.

Cut edge browning in shredded cabbage could therefore be attributed to the enzymatic reactions in response to wounding following alteration in PAL activity. While some authors have previously reported γ-irradiation induced browning inhibition in cut vegetables, the present work provides a comprehensive insight into the mechanism of browning inhibition at both a biochemical and a genetic level for the first time.

4. Conclusion

The current work demonstrated the feasibility of radiation processing as an effective post harvest processing method in inhibiting cut edge browning in shredded cabbage. Thus, besides being a highly effective method for ensuring food safety, γ-irradiation provides an improved benefit in terms of maintaining visual quality of the product.

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Allyl isothiocyanate enhances shelf life of minimally processed shredded cabbage

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ABSTRACT

The effect of allyl isothiocyanate (AITC), in combination with low temperature (10 °C) storage on post harvest quality of minimally processed shredded cabbage was investigated. An optimum concentration of 0.05 μL/mL AITC was found to be effective in maintaining the microbial and sensory quality of the product for a period of 12 days. Inhibition of browning was shown to result from a down-regulation (1.4-fold) of phenylalanine ammonia lyase (PAL) gene expression and a consequent decrease in PAL enzyme activity and o-quinone content. In the untreated control samples, PAL activity increased following up-regulation in PAL gene expression that could be linearly correlated with enhanced o-quinone formation and browning. The efficacy of AITC in extending the shelf life of minimally processed shredded cabbage and its role in down-regulation of PAL gene expression resulting in browning inhibition in the product is reported here for the first time.

1. Introduction

Cabbage (*Brassica oleracea* var *capitata*) is a leafy green vegetable of brassica family, grown as an annual vegetable crop worldwide. Fresh leaves of cabbage are used for preparation of a wide variety of recipes including delicacies like sauerkraut and kimchi. It is a rich source of phenolics, glucosinolates, vitamins and minerals and is known for its nutritional value. Cabbage is also widely marketed as a minimally processed (MP) product in the shredded form due to the associated convenience and fresh characteristics. Post harvest loss due to microbial decay and physiochemical changes tend to decrease post harvest shelf life of minimally processed products. A number of chemical preservatives are usually applied for preservation of such products.

In recent years there has been considerable demand from consumers to reduce or eliminate chemically synthesized additives in foods (Lanciotti et al., 2004). Plant derived products represent a source of natural preservatives to improve the shelf-life and the safety of food. In this regard, plant products, including essential oils that are biodegradable and eco-friendly, have received increasing attention in recent years. Such products are effective, economical, and environmentally safe and can be ideal candidates for use as agrochemicals. Many biologically active volatile compounds such as allyl isothiocyanate (AITC), (E)-2-hexenal (Fallik et al., 1998; Archbold, Hamilton-Kemp, Barth, & Langlois, 1997), hexanal (Gardini, Lanciotti, Caccioni, & Guerzoni, 1997), and methyl jasmonates (González-Aguilar, Buta, & Wang, 2003) have shown potential to inhibit the growth of postharvest microbial flora and reduce postharvest diseases. Literature data indicate that these aroma compounds can represent a useful tool to increase shelf-life of minimally processed fruits. AITC, 2-hexenal, hexanal, methyl jasmonate, eugenol, menthol and thymol have been used to increase the shelf life of fruits like strawberries, apples and blueberries (Wang, Chen, & Yin, 2010). Shik Shin et al. (2010) and Ko, Kim, and Park (2012) have reported the use of AITC for increasing the shelf life of fermented products like tofu and kimchi. However, studies on the use of these compounds for preservation of minimally processed fresh cut vegetables are limited. A detailed investigation in this direction can prove beneficial to food industry for preservation of fresh cut vegetables. While the aroma compounds of plant origin are generally recognized as safe (GRAS), their use is often limited due to a high impact on the organoleptic characteristics of the food products. Hence, use of volatiles which are natural ingredients of the product itself is highly recommended as they are compatible with the overall organoleptic quality of the product.

AITC is known to be a major volatile aroma constituent of cabbage responsible for its characteristic flavor (Buttery, Guadagni, Ling, Seifert, & Lipton, 1976). The compound is also reported to

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possess various biological activities including antioxidant and antimicrobial properties (Shapiro, Fahey, Wade, Stephenson, & Talalay, 2001). Nagata (1996) have earlier shown that exogenous application of AITC to shredded cabbage resulted in browning inhibition at the cut edges for 48 h. While these authors proposed a decrease in PAL and PPO activities during such a treatment, the mechanism at molecular level was not investigated. The present work therefore aims to evaluate the efficacy of AITC in extending the shelf life of minimally processed shredded cabbage and understand the role of this compound in browning inhibition at molecular level.

2. Materials and methods

2.1. Plant material

Fresh cabbage (*B. oleracea*, BC-79 variety) samples were obtained from Dr. Panjabrao Deshmukh Agricultural University, India. Harvesting was done 65 days after planting when the above variety is known to be mature. They were cut with sterile stainless steel knives into 1 cm wide by 3.0–3.5-cm long strips. Cut samples were packed (40 g) into polystyrene trays (id: 9 cm × 9 cm × 2.5 cm).

2.2. Treatment and storage

Treatment with volatile compound was done according to the protocol followed by Wang et al. (2010). Briefly, AITC (0.005 μL/mL, 0.01 μL/mL, 0.05 μL/mL and 0.1 μL/mL; volume of sample/volume of the tray) was spotted onto a piece of filter paper which was subsequently placed inside the trays just before the trays were overwrapped with cling film. The volatiles were allowed to vaporize inside the containers spontaneously at 20 °C for 16 h. The containers were then stored at 10 °C.

2.3. Microbial analysis

Standard methods were used to enumerate microorganisms present in MP cabbage at each sampling time and treatment for 21 days of storage. Mesophilic bacteria, yeast, and mold counts were carried out according to the method described by Saroj et al. (2006). Sample (25 g) was homogenized in 225 mL of sterile physiological saline. After appropriate serial dilutions, the samples were pour plated on plate count agar. The colonies were counted after 24 h of incubation at 37 °C. Total yeast and mold counts were performed with the pour plate method using potato dextrose agar supplemented with 0.1% tartaric acid to maintain media pH at 3.5. Plates were incubated at 37 °C for 48 h. Microbial counts were expressed as $\log_{10}$ CFU g^{-1} of vegetable.

2.4. Sensory analysis

Sensory analysis was carried out by a sensory panel of 15 members (7 women and 8 men). The panelists were chosen according to following criteria: people with no food allergies, nonsmokers, with age between 25 and 55 y, available for all sessions and interested in participating. All panelists had previous experience in carrying out sensory analysis of similar food products. Samples for sensory evaluation were prepared by boiling MP cabbage in water and immediately cooling it in chilled water. Boiling time was chosen in preliminary experiments by serving samples boiled for different duration (2 min, 5 min and 10 min) to the panelist. Amongst these the samples boiled for 5 min was liked most. Cabbage samples (10 g) were served in white trays numbered randomly to the sensory panel. The replicates were assessed in three different sessions to avoid tiredness and saturation. The panelists had to consume the whole sample and rinse their mouths with water between them.

Hedonic test was carried out using a 9-point scale with 1, dislike extremely or not characteristic of the product and 9, like extremely or very characteristic of the product (Lopez-Rubira, Conesa, Allende, & Artes, 2005). Parameters evaluated were color, aroma, texture, taste and overall acceptability. To determine the acceptability of the samples at different storage points, all the parameters analyzed were compared with fresh control samples on each day. The scores given for all the attributes for each sample were tabulated. The mean value was calculated for each attribute of a sample that represented the panel's judgment about the sensory quality of the product and significant difference was found by analysis of variance (ANOVA).

2.5. Evaluation of browning

Browning of the cut edges was measured by Minolta Chromameter (model CM-3600d Konica Minolta Sensing Inc., Japan). Instrument calibration was done with a white tile supplied with it and then used to determine the color using the 3 Commission Internationale de l'Eclairage (CIE) coordinates, L (lightness), a (−green, +red), and b (−blue, +yellow). Nine strips of cabbage were selected randomly from each packaged tray and results represent their average.

2.6. PAL assay

PAL activity was measured according to Degl'innocenti, Guidi, Pardossi, and Tognoni (2005) with some modifications. 10 g sample was homogenized with 30 mL of cold borate buffer (50 mM, pH 8.5) containing 5 mM 2-mercaptoethanol and 0.2 g of PVPP. The homogenate was filtrated through 4 layers of cheesecloth and centrifuged at 12,000 rpm at 4 °C for 20 min. PAL activity was measured after the addition of 2 mL of 50 mM L-phenylalanine to 1 mL of the supernatant and incubation at 40 °C for 1 h. The absorbance was measured at 290 nm before and after incubation. One unit of PAL activity equals the amount of PAL that produced 1 μmol of *trans*cinnamic acid in 1 h; it is expressed as μmol g^{-1} FW h^{-1}.

2.7. PPO assay

PPO extraction was done according to Degl'Innocenti et al. (2005). The standard reaction mixture consisted of 250 μL of 0.2 M sodium phosphate buffer (pH 6.0), 50 μL of 1.0 M catechol, and 50 μL of enzyme extract. The reaction was carried out at 30 °C for 5 min, and PPO activity was measured by monitoring the increase in absorbance at 420 nm. One unit of PPO activity was defined as the amount of enzyme that caused an increase in absorbance of 0.01 per minute.

2.8. POD assay

Extraction procedure followed was same as for POD assay. The chlorogenic acid peroxidase assay contained 800 μL of 50 mM potassium phosphate buffer, pH 6.5, 50 μL of 80 mM chlorogenic acid, 50 μL of extract, and 100 μL of 35 mM H_2O_2. The caffeic acid peroxidase assay contained 800 μL of McIlvaine (114 mM Na2HPO4 and 43 mM citric acid), pH 5.5, 50 μL of 80 mM caffeic acid, 50 μL of extract, and 100 μL of 35 mM H_2O_2. In all cases, POD assays were initiated by the addition of H_2O_2. Absorbance was measured at 410 nm for chlorogenic acid and 470 nm for caffeic acid peroxidase activity. The activities of PODs are expressed as ΔA_λ min^{-1} g^{-1} fresh weight.

2.9. o-Quinone content

Soluble o-quinones of leaf vegetable tissues were extracted as described by Ke and Saltveit (1986). 10 g of tissue were homogenized with 20 mL of methanol. The homogenate was filtered through four layers of cheesecloth and centrifuged at 12,000 rpm for 20 min. The supernatant was used directly to measure the soluble o-quinones at a wavelength of 437 nm.

2.10. RNA extraction and cDNA preparation

RNA extraction was done using TRI reagent (Sigma, T 9424) as per the manufacturer's instructions. The quantity of RNA was measured using a NanoDrop 3300 spectrophotometer (Thermo Scientific, MA) and the integrity was checked by electrophoresis of total RNA (1 μg) on a 1.2% denaturing agarose gel (Vincze & Bowra, 2005).

1 μg of the total RNA was reverse transcribed with Superscript™ III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen, USA) as per manufacturer's instructions.

2.11. Quantitative real time-PCR

Previously reported primer sets of PAL (*PAL1*) and actin (*ACTIN2*) as reference gene for normalization and quantification were used for qRT-PCR (Sup Table 1) (Srivastava, Ramaswamy, Suprasanna, & D'Souza, 2010). qRT-PCR was carried out using a Corbett rotor gene 3000 (Corbett Life Science, www.corbettlifescience.com). Detection of real-time RT-PCR products was done using a SyBr Green Master Mix kit (S 4320, Sigma), as per the manufacturer's instructions. The PCR cycling conditions comprised an initial cycle at 50 °C for 2 min followed by one cycle at 95 °C for 10 min and 40 cycles each comprising 95 °C for 30 s, 55 °C for 45 s, and 72 °C for 30 s. For each sample, reactions were set up in triplicate to ensure the reproducibility of the results.

2.12. Texture analysis

The texture analysis for the sample was performed using a Texture Analyzer (TA. HD. Plus, Stable Micro Systems). Puncture strength of the strips (1 cm × 3 cm) were determined by 2 mm needle probe having test speed of 30 mm/min.

2.13. Preparation of methanolic extracts

Forty grams of cabbage were extracted twice with 150 mL of aqueous methanol. The extract was filtered (whatman filter 1) and the supernatant concentrated in a flash evaporator (Buchi Rotavapor R114) to make a 1% solution that was used for subsequent assays.

2.14. DPPH radical scavenging activity

A DPPH radical scavenging assay was used to evaluate total antioxidant activity of cabbage (Jao & Ko, 2002). An aliquot of methanolic extract (100 μL) was added to 1 mL of DPPH solution (110 μM in 80% aq methanol). After incubation under dark conditions for 20 min absorbance was measured at 516 nm. DPPH radical scavenging activity was expressed as the μg gallic acid equivalent (GAE)/g of cabbage.

2.15. Total phenolic content

Total phenolic content (TPC) was evaluated in accordance with the Folin–Ciocalteu procedure (Singleton & Rossi, 1965). TPC in cabbage was expressed as mg GAE 100 g^{-1} fresh weight (FW) of cabbage.

2.16. Vitamin C content

Total vitamin C content of cabbage was estimated in accordance with standard AOAC official titrimetric method (AOAC, 1990). Cabbage (10 g) was extracted with 20% metaphosphoric acid in an omnimixture. The homogenate was centrifuged at 12,000 rpm for 20 min. Reducing capacity of the supernatant was measured by titrating with 2,6 dichlorophenol indophenol. The end point of the reaction was detected by appearance of pink color by excess of the dye in the acidic solution. The same process was followed for standard ascorbic acid solutions of known concentration (0.1–0.0015%). Ascorbic acid content was expressed as mg/100 g FW of cabbage.

Table 1
Effect of AITC treatment on different sensory parameters of MP cabbage.

Day	Concentration (μL/mL)	Color	Texture	Taste	Aroma	Over all acceptability
0	0	7.1 ± 1.1^{a}	7.2 ± 1.2^{a}	6.9 ± 1.2^{a}	6.8 ± 0.4^{a}	7.1 ± 1.2^{a}
	0.005	7.1 ± 1.1^{a}	7.2 ± 1.2^{a}	6.9 ± 1.2^{a}	6.8 ± 0.4^{a}	7.1 ± 1.2^{a}
	0.01	7.1 ± 1.1^{a}	7.2 ± 1.2^{a}	6.9 ± 1.2^{a}	6.8 ± 0.4^{a}	7.1 ± 1.2^{a}
	0.05	7.1 ± 1.1^{a}	7.2 ± 1.2^{a}	6.9 ± 1.2^{a}	6.8 ± 0.4^{a}	7.1 ± 1.2^{a}
	0.1	7.1 ± 1.1^{a}	7.2 ± 1.2^{a}	6.9 ± 1.2^{a}	6.8 ± 0.4^{a}	7.1 ± 1.2^{a}
5	0	5.3 ± 0.1^{b}	7.1 ± 1.1^{a}	6.1 ± 2.1^{a}	6.6 ± 1.2^{a}	5.1 ± 0.3^{b}
	0.005	5.8±.1.2^{ab}	7.2 ± 1.2^{a}	6.2 ± 1.1^{a}	6.2 ± 0.8^{a}	5.8 ± 0.6^{b}
	0.01	7.2 ± 1.5^{a}	6.8 ± 2.1^{a}	6.4 ± 2.2^{a}	6.3 ± 0.4^{a}	6.8±.1.2^{a}
	0.05	7.9 ± 1.1^{a}	6.2 ± 1.6^{a}	6.1 ± 1.8^{a}	6.1 ± 1.1^{ab}	7.2 ± 1.5^{a}
	0.1	7.1 ± 1.4^{a}	6.7 ± 1.2^{a}	6.1 ± 2.3^{a}	5.1 ± 0.2^{b}	4.9 ± 1.1^{b}
8	0	3.6 ± 1.1^{b}	6.4 ± 2.7^{a}	NA	6.1 ± 0.8^{a}	3. 2 ± 0.5 ^{c}
	0.005	5.1 ± 0.8^{b}	6.8 ± 2.1^{a}	6.2 ± 1.6^{a}	6.3 ± 1.1^{ab}	3.4 ± 0.7 ^{c}
	0.01	7.2 ± 0.3^{a}	7.1 ± 1.2^{a}	6.2 ± 1.8^{a}	6.1 ± 1.4^{b}	5.1 ± 0.6^{b}
	0.05	7.6 ± 1.1^{a}	6.6 ± 1.4^{a}	6.2 ± 1.9^{a}	6.8 ± 0.8^{b}	7.2 ± 0.3^{a}
	0.1	7.9 ± 1.1^{a}	6.5 ± 1.2^{a}	6.2 ± 1.9^{a}	4.7 ± 1.3 ^{c}	4.6 ± 0.4^{b}
12	0	2.1 ± 0.9 ^{c}	6.3 ± 0.1^{a}	NA	4.2 ± 1.5^{bcd}	3.1 ± 0.8 ^{c}
	0.005	2.5 ± 1.2 ^{c}	6.1 ± 0.3^{a}	NA	4.1 ± 0.1 ^{d}	3.1 ± 0.3 ^{c}
	0.01	3.2 ± 1.3^{bc}	6.1 ± 0.4^{a}	6.2 ± 2.4^{a}	5.1 ± 0.1 ^{c}	3.8 ± 0.9 ^{c}
	0.05	7.1 ± 1.2^{a}	6.1 ± 0.3^{a}	6.6 ± 2.1^{a}	6.2 ± 0.2^{a}	7.1 ± 0.2^{a}
	0.1	7.4 ± 1.1^{a}	6.5 ± 0.5^{a}	6.8 ± 1.6^{a}	4.1 ± 0.2^{b}	4.2 ± 1.3^{b}

Data are expressed as mean ± standard deviation (n = 9). Mean values in the same column for each day bearing same superscript shows no significant difference ($p \leqslant 0.05$).

2.17. Statistical analysis

DSAASTAT ver. 1.101 by Andrea Onofri was used for statistical analysis of data. Data was analyzed by Analysis of variance (ANOVA) and multiple comparisons of means were carried out using Duncan's multiple range test. Data are expressed as means ± SD of three independent analyses each carried out in triplicate unless otherwise mentioned. Means are expressed as significantly different or not at 5% level of confidence.

3. Results

3.1. Microbial analysis

Effect of volatile treatment on microbial load is shown in Fig. 1. A significant ($p \leqslant 0.05$) increase in bacterial load during storage was observed in the control samples wherein the bacterial counts were higher than 10^7 CFU/g on day 8 which is beyond the acceptable limit (10^7 CFU/g) prescribed for fresh cut vegetables and fruits (Oms-Oliu, Aguilo-Aguayo, Martin-Belloso, & Soliva-Fortuny, 2010 and Gilbert et al., 2000) (Fig. 1A). Compared to the control, AITC treatment inhibited the growth of microbial flora in minimally processed cabbage. A concentration dependent decrease in microbial load during storage was noted (Fig. 1A). In the treated samples (0.05 μL/mL and 0.1 μL/mL), the mesophilic counts remained well below the acceptable limit up to a storage period of 12 days at 10 °C. The response of yeast and mold count at different concentrations of AITC is shown in Fig. 1B. Control samples showed an increase in fungal count with storage. A dose dependent decrease in fungal population was noted in the treated samples with fungal count remaining below 10^7CFU/g up to a storage period 12 days in samples treated with 0.05 μL/mL and 0.1 μL/mL of the volatile.

Thus AITC could effectively maintain the microbial safety of the minimally processed shredded cabbage up to 12 days at 10 °C.

3.2. Sensory analysis

Table 1 demonstrates the effect of volatile treatment and storage on different sensory attributes, viz, appearance, aroma, texture and taste on cabbage samples. Sensory quality of control samples was found to deteriorate within 3 days due to browning of the cut edges. This increased to a high level on day 7. Samples treated with 0.005 and 0.01 μL/mL of AITC showed significant blackening at the end of storage period. However, those treated with higher concentrations (0.05 and 0.1 μL/mL) of AITC appeared fresh throughout the storage period.

Since the present study involves preservation of shredded cabbage using aroma compounds, aroma quality of the samples forms an important parameter. In control samples a significant decrease in aroma quality was observed beyond 8 days of storage. The treated samples however showed difference in aroma quality depending on the concentration of volatile used. The samples treated with highest concentration of AITC (0.1 μL/mL) had slightly harsh odor throughout the storage period. Samples treated with lower doses (0.005 and 0.01 μL/mL) of AITC retained good aroma quality up to 10 days of storage. The samples treated with 0.05 μL/mL of AITC, however, received good aroma scores throughout the storage period.

No difference in texture and taste scores was perceived by the sensory panel between the control and treated samples at all concentration. Thus based on aroma and visual quality, 0.05 μL/mL of AITC was found to be the optimum concentration of the compound that increased the shelf life of shredded cabbage by 5 days at 10 °C.

3.3. Texture analysis

Fig. 1C provides the puncture strength of both the control and treated samples. The firmness of the control and all the treated samples remained unaffected throughout the storage period of 12 days. The data obtained are in agreement with the scores on texture obtained from the sensory panel.

3.4. Evaluation of browning

Fig. 1D represents the effect of AITC treatment and storage on *L* values of MP cabbage strips. A continuous decrease in *L* values during storage, with a substantial decrease beyond day 3 was observed in the control sample. Samples treated with lower

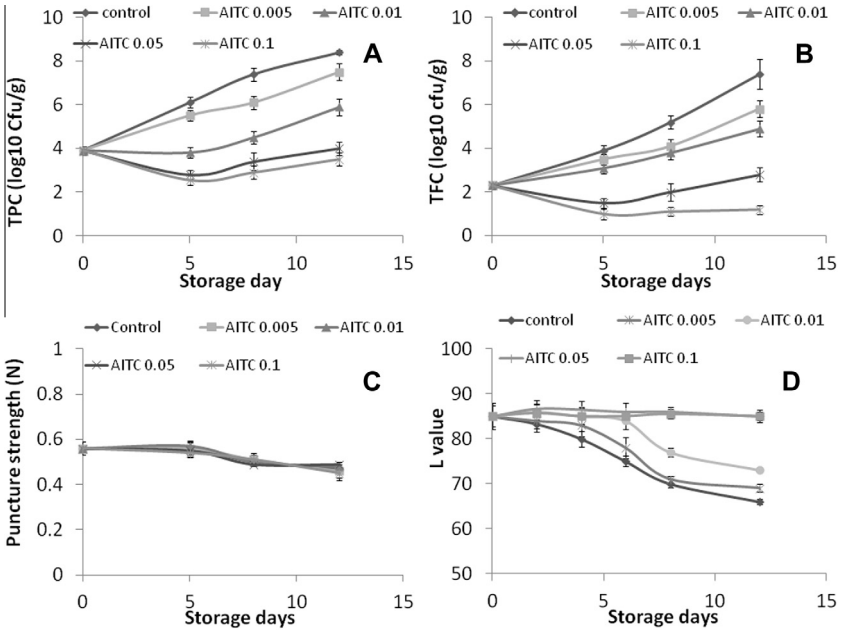

Fig. 1. (A) Total plate count (TPC) of AITC treated minimally processed shredded cabbage during storage. (B) Total fungal count (TFC) count of AITC treated minimally processed shredded cabbage during storage. (C) Effect AITC treatment and storage on puncture strength of minimally processed shredded cabbage. (D) Effect AITC treatment and storage on L value of minimally processed shredded cabbage. Values are expressed as mean ± SD ($n = 9$).

concentration (0.005 and 0.01 μL/mL) of AITC revealed a decrease in *L* value from day 5 onwards. Interestingly, luminosity (*L*) of the cabbage strips treated with higher concentration of AITC (0.05 and 0.1 μL/mL) remained unchanged during storage and the visual quality was acceptable at the end of the storage period.

3.5. Evaluation of enzyme activity

Since AITC treatment could effectively inhibit browning in MP cabbage throughout the storage period, the activities of the different enzymes associated with browning in cabbage were studied at different storage intervals. Fig. 2A illustrates the effect of AITC treatment on PAL activities at different storage time. An increase in PAL activity was observed in control samples with storage. In the treated samples, however, a concentration dependent decrease in PAL activity was noted. Samples treated with 0.005 μL/mL showed an increase in PAL activity from day 3 onwards while samples treated with 0.01 μL/mL AITC showed increased PAL activity from day 6 onwards. In the 0.05 μL/mL and 0.1 μL/mL treated samples, an initial decrease was noted in PAL activity followed by slight increase that further remained constant. PPO and POD activities were also monitored throughout the storage period of 12 days. No change in PPO and POD activity was noted in the control and treated sample throughout the storage period (Table 2).

3.6. Real-time PCR analysis of PAL gene

Since PAL was the only enzyme affected by AITC treatment, transcriptional analysis of PAL gene at different storage points was studied. The minimum concentration required to inhibit cut edge browning up to a storage period of 12 days was found to be 0.05 μL/mL. Hence, gene expression was analyzed for control samples and samples treated with 0.05 μL/mL of AITC (Fig. 3). Gene expression was analyzed at 0, 24 and 48 h of storage. The control sample showed a gradual increase in PAL gene expression with storage. An up-regulation of 1.2-fold and 7.7-fold was seen after 24 h and 48 h respectively. In AITC treated samples a small but significant down-regulation was observed after 24 h of storage which however was found to revert back to basal value by 48 h thus maintaining a low but constant level of PAL activity in these samples.

3.7. o-Quinone content

A concentration dependent decrease in o-quinone content was noted in treated samples (Fig. 2C). o-Quinone content was observed to increase in samples treated with 0.005 μL/mL and 0.01 μL/mL AITC from day 5 and day 8 onwards respectively. No change in its content was, however, noted in the samples treated with 0.05 μL/mL and 0.1 μL/mL of AITC.

3.8. Total phenolic content and radical scavenging activity

No change in total phenolic content was noted between control and treated samples up to 8 days of storage. However, beyond this period, a significant decrease was noted in samples treated with 0.05 μL/mL and 0.1 μL/mL of AITC (Fig. 4A). No change in the DPPH radical scavenging activity was noted in both control and treated samples throughout the storage period of 12 days (Fig. 4B).

3.9. Vitamin C content

Variation in the vitamin C content among different cabbage cultivars ranging from 5.7 to 23.5 mg/100 g has been reported by Singh, Upadhyay, Prasad, Bahadur, and Rai (2007). The amount estimated in present study is in agreement with the previous reports available. No change in the vitamin C content was observed in both the control and treated samples throughout the storage period of 12 days (Fig. 4C).

4. Discussion

Microbial decay is one of the major causes of rapid post-harvest deterioration of fresh produce. Use of natural antimicrobials such as plant volatiles to combat microbial growth has recently gained increased importance in the area of preservation of fresh produce. In the present study treatment with AITC (0.01, 0.05 and 0.01 μL/

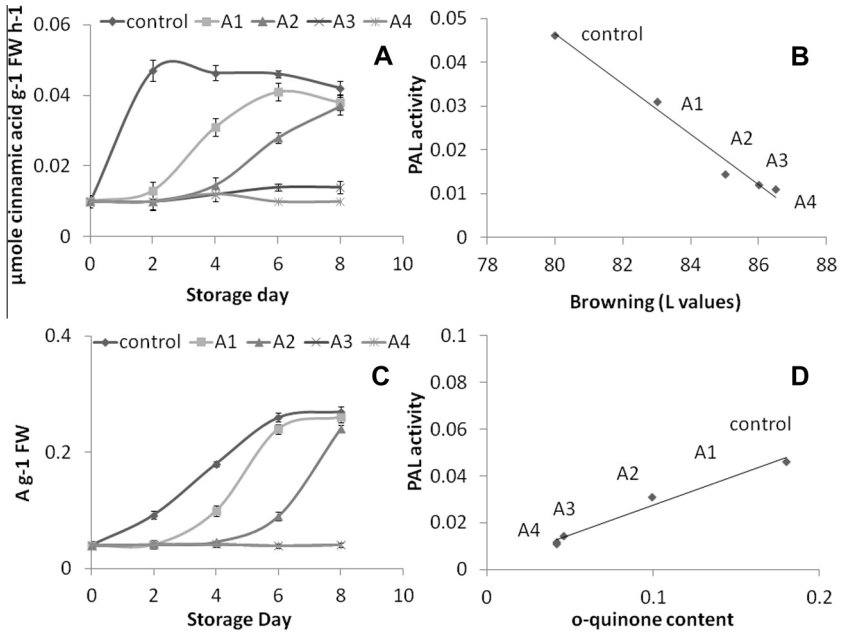

Fig. 2. (A). Effect of AITC treatment and storage (8 days) on PAL activity. (B) Plot depicting the relation between PAL activity and browning in shredded cabbage treated with different concentrations of AITC. (C) Effect of AITC treatment and storage (8 days) on o-quinone content. (D) Plot depicting the relation between PAL activity and o-quinone content in shredded cabbage treated with different concentrations of AITC. A1 = 0.005 μL/mL, A2 = 0.01 μL/mL, A3 = 0.05 μL/mL, A4 = 0.1 μL/mL.

Table 2
Effect of AITC treatment on PPO and POD activities.

Day	Control			0.01 μL/mL			0.05 μL/mL			0.1 μL/mL		
	PPO	POD1	POD2	PPO	POD1	POD2	PPO	POD1	POD2	PPO	POD1	POD2
0	10.1 ± 2.1[a]	5.1 ± 1.1[a]	3.4 ± 2.2[a]	11.2 ± 1.2[a]	6.1 ± 1.1[a]	4.8 ± 1.8[a]	11.1 ± 3.2[a]	6.1 ± 2.1[a]	5.1 ± 1.1[a]	11.1 ± 3.1[a]	6.1 ± 1.2[a]	4.6 ± 1.1[a]
2	10.1 ± 3.7[a]	6.2 ± 2.2[a]	5.1 ± 1.8[a]	10.5 ± 4.1[a]	5.7 ± 1.4[a]	6.1 ± 1.2[a]	12.2 ± 2.2[a]	6.2 ± 1.9[a]	5.2 ± 1.2[a]	12.1 ± 3.1[a]	5.9 ± 2.1[a]	6.1 ± 2.1[a]
4	11.4 ± 2.1[a]	6.3 ± 1.1[a]	4.5 ± 2.6[a]	11.1 ± 2.2[a]	5.1 ± 2.4[a]	5.8 ± 1.2[a]	10.5 ± 2.2[a]	5.8 ± 1.4[a]	4.9 ± 2.1[a]	11.1 ± 3.3[a]	6.1 ± 2.3[a]	5.1 ± 1.3[a]
6	11.1 ± 2.1[a]	5.8 ± 3.1[a]	4.1 ± 1.5[a]	11.7 ± 2.1[a]	5.1 ± 2.1[a]	5.8 ± 2.3[a]	12.1 ± 4.7[a]	6.2 ± 1.3[a]	5.2 ± 0.7[a]	12.5 ± 3.1[a]	6.7 ± 1.9[a]	6.1 ± 1.5[a]
8	10.2 ± 3.9[a]	5.4 ± 2.3[a]	5.8 ± 2.1[a]	10.3 ± 2.1[a]	6.4 ± 1.2[a]	5.1 ± 1.7[a]	10.9 ± 2.3[a]	6.3 ± 2.2[a]	4.9 ± 1.1[a]	10.1 ± 2.1[a]	6.8 ± 2.1[a]	5.9 ± 1.3[a]

Data are expressed as mean ± standard deviation ($n = 9$). Mean values in the same column bearing same superscript shows no significant difference ($p \leqslant 0.05$). PPO activity is represented in U g^{-1} FW, POD activity is represented in ΔA min^{-1} g^{-1} FW; POD1 is caffeic acid peroxidase activity and POD2 is chlorogenic acid peroxidase activity.

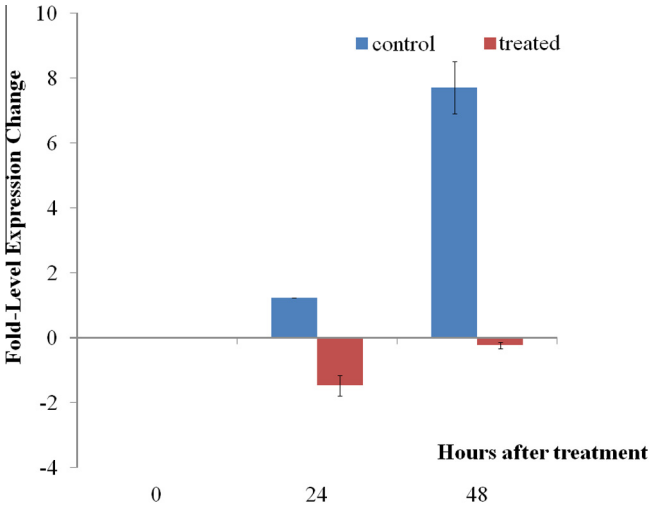

Fig. 3. Fold change in the expression of PAL gene. The *x*-axis represents the expression level of PAL gene in control at 0 h. All values are means of triplicates ±SD.

mL) was found to restrict microbial growth within the acceptable limit in minimally processed shredded cabbage throughout the storage period of 12 days at 10 °C. To check the stability of AITC in the packets the AITC content of the individual packets were monitored for a period of 12 days by GC/MS headspace analysis (Supplementary Table 1). The AITC content of the packets at the end of the storage period was found to decrease by only 2.6% as compared to day 0. Hence, cling film was confirmed to be successful in retaining AITC in the packaged samples. AITC has been demonstrated to possess strong antimicrobial activity against *Escherichia coli, Salmonella typhimurium, Pseudomonas aeruginosa, Listeria monocytogenes, Staphylococcus aureus* and other pathogenic bacteria (Liu & Yang, 2010). However, the precise mechanism of its action is yet unclear. Many reports indicate the cell membrane to be the primary target of bioactive aroma compounds. Membrane disruption by aroma compounds has been observed in both bacteria and fungi (Cox et al., 2000; Helander, von Wright, & Matilla-Sandholm, 1997). AITC is known to generate ROS like H_2O_2 during storage which causes microbial DNA damage resulting in bactericidal activity (Wang et al., 2010).

AITC was found to be effective in maintaining microbial safety of minimally processed cabbage throughout the storage period. However, the use of volatile aroma compounds is often limited because of their high impact on the organoleptic characteristics of food products. Hence, sensory quality of the treated samples thus forms an important parameter deciding their consumer acceptability. The samples treated with 0.05 and 0.1 μL/mL of AITC demonstrated excellent visual quality throughout the storage period. AITC is known to possess characteristic cabbage like pungent aroma which may influence the aroma quality of the products. Hence, determining the optimum concentration of the volatile that can maintain the microbial quality of the samples without lowering the aroma quality is crucial. The 0.1 μL/mL treated sample was disliked by the panelist owing to its harsh aroma. On the other hand 0.05 μL/mL treated samples received good aroma scores throughout.

Appearance of the product is another important parameter deciding consumer acceptance of the product. Cut edge browning often forms the major factor that affects appearance of fresh products. AITC could effectively inhibit browning in MP cabbage throughout the storage period of 12 days. The antibrowning effect of AITC on cabbage has been previously reported by Nagata (1996) wherein AITC was found to inhibit cut edge browning in cabbage for 48 h due to inhibition of activity of enzymes such as PAL and PPO. In the present study AITC inhibited cut edge browning in cabbage up to a period of 12 days. Hence, the effect of different concentrations of AITC on activities of the various enzymes involved in browning was of interest.

In our previous study on post harvest physiology of shredded cabbage, cut edge browning on storage was demonstrated to be due to an alteration in enzyme activities (Banerjee, Penna, Variyar, & Sharma, 2015). PAL is the first enzyme in the phenylpropanoid pathway involved in synthesis of phenolic compounds. An increase in PAL activity was observed in the control samples in the present study. A consequent up-regulation of PAL gene expression (1.2-fold) within 24 h of shredding and a further increase by 7.7-fold on storage up to 48 h was also noted. Similar results were seen in cut lettuce wherein a 3.4-fold increase in PAL mRNA expression was reported within 24 h of wounding resulting in an increase in PAL activity (Campos-Vargas, Nonogaki, Suslow, & Saltveit, 2005). An enhanced synthesis of phenolic compounds in the control samples as a result of shredding and a consequent increase in browning intensity with storage could thus be inferred. Treatment of the samples with AITC at concentrations of 0.05 μL/mL and 0.1 μL/mL completely inhibited browning in the stored product. In these samples an initial decrease in PAL activity was followed by a slight increase that that further remained constant throughout the storage period. AITC at a concentration of 0.05 μL/mL was found to be the minimum concentration required to inhibit cut edge browning for a storage period of 12 days. Hence, PAL gene expression was monitored only in samples treated with 0.05 μL/mL of AITC. A slight down-regulation was seen after 24 h of storage that, however, reverted back to basal value within 48 h. Thus a nearly constant and low level of PAL activity was observed in the treated samples. PPO and POD are the downstream enzymes that oxidize phenolic compounds to brown pigments. In the present study, PPO and POD activities were unaffected as a result of AITC treatment during the entire storage period. Similar, results were obtained in irradiated cabbage and heat treated lettuce wherein no change in PPO and POD activities

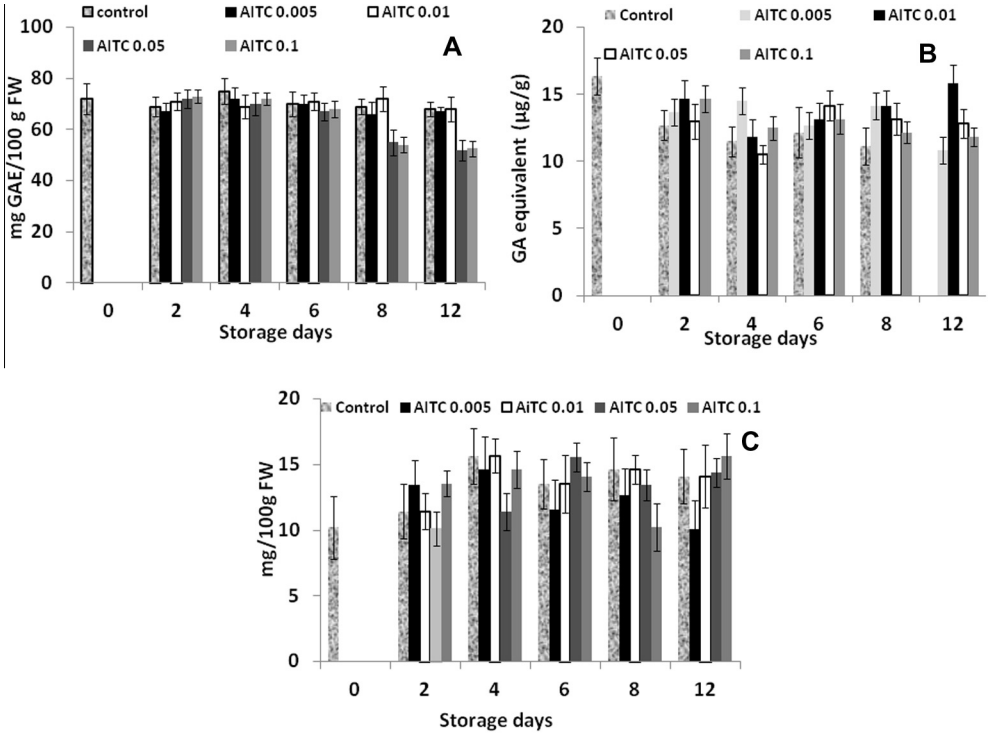

Fig. 4. Effect of AITC treatment and storage on A. Total phenolic content; B. DPPH radical scavenging activity; C. Vitamin C content. Values are expressed as mean ± SD (n = 9).

were observed due to treatment or storage. Further, a negative correlation (R^2 = −0.98) between PAL activity and browning (Fig. 2B) confirmed the decrease in PAL activity to be the key factor for browning inhibition in AITC treated samples.

As AITC was found to decrease PAL activity, it was of interest to determine the variation in the phenolic content during such a treatment. No change in phenolic content was observed up to a storage period of 8 days beyond which a slight but significant decrease in its content was noted. Similar results have been obtained by Wang et al. (2010) wherein AITC treatment of blueberries was found to decrease the phenolic content on storage. No correlation could be established between phenolic content and browning intensity in the present study. Our results are in accordance with the reports of Hisaminato, Murata, and Homma (2001) and Vitti, Sasaki, Miguel, Kluge, and Morett (2011) for cut lettuce and potato respectively wherein no correlation between phenolic content and browning was demonstrated. However, the quinone content showed a good correlation (R^2 = 0.99) with PAL activity (Fig. 2D) as well as with browning, thereby establishing a direct relationship between decrease in PAL activity and browning inhibition in the treated samples. A lowering in PAL activity in radiation processed shredded cabbage (Banerjee et al., 2015) and heat shock treated lettuce (Vitti et al., 2011) resulting in browning inhibition has been previously reported. Many authors have claimed PAL activity to be an index of deterioration of fresh-cut products during processing particularly with respect to their color and texture.

Loss in firmness of vegetables can affect consumer acceptability. No change in texture was noted in the control samples throughout the storage period. Volatile treatment also did not affect the texture of the samples. Similar results were obtained by Song, Fan, Forney, Campbell-Palmer and Fillmore (2010) in Brigitta blueberries treated with volatile aroma compounds wherein no change in texture was obtained due to volatile treatment and storage.

Vitamin C is a major nutritional constituent present in fresh fruits and vegetables. It acts as an antioxidant in the body by protecting against oxidative stress and is also a cofactor in several key enzymatic reactions. Vitamin C is also the most sensitive vitamin being degraded quickly on exposure to heat, light and oxygen. The content of vitamin C was found to be unaffected by AITC treatment and storage in the present study. Fresh fruits and vegetables are also known to possess considerable antioxidant properties. However, processing operations and storage tend to decrease the inherent antioxidant properties of these products. The DPPH test is usually used to provide basic information on the overall antioxidant property of samples. In the present studies DPPH radical scavenging activity was also found to remain unchanged in control as well as treated samples throughout the storage period of 12 days. AITC treatment could thus maintain the nutritional quality with respect to vitamin C and antioxidant activity throughout the storage period of 12 days.

5. Conclusion

The present study has demonstrated the efficacy of AITC to serve a dual purpose of both microbial and sensory quality of MP cabbage thereby increasing its shelf life. Further, unlike the earlier literature report (Nagata, 1996) on the decreased PAL and PPO activities during AITC treatment, our results clearly showed that decreased PAL activity alone was responsible for the browning inhibition during such a treatment. This is the first report on the application of volatile aroma compounds for enhancing the post harvest shelf life of fresh cut vegetable like cabbage. AITC being almost ubiquitously present in brassica vegetables can be used for other brassica vegetables also. Owing to its natural origin AITC can thus prove beneficial to food industry for preservation of fresh cut vegetables.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.03.063.

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CHAPTER

Role of Glucosinolates in Plant Stress Tolerance

12

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12.1 Introduction

Plants elicit multiple responses when exposed to a complex array of biotic (e.g., pathogen infection and herbivore feeding) and abiotic (e.g., nutrient levels and light conditions) stress factors. These stress factors induce signaling cascades that activate ion channels, kinases, production of reactive oxygen species (ROS), and accumulation of hormones such as salicylic acid (SA), ethylene (ET), jasmonic acid (JA) and abscisic acid (ABA) (Mittler, 2006; Jain, 2013). These signals eventually induce expression of specific subsets of defense genes that produce an overall defense response (Mittler et al., 2004; Mantri et al., 2011; Arbona et al., 2013). Activation of defense systems affects both the primary and secondary metabolism resulting in a substantial and significant variation in plant metabolome within and between species. Chemical defenses form a part of the plant's inherent immune system. Plants produce a wide diversity of secondary metabolites that play a prominent role in defense against herbivores and pathogens. Some of them also act as defense against abiotic stress and in communication with its own species and with other organisms. Plant secondary chemistry is phenotypically plastic and varies in response to both biotic and abiotic factors. The major classes of secondary metabolites include the terpenoids and phenolics as well as the nitrogen (N)- and sulfur (S)-containing compounds synthesized primarily from amino acids.

Brassicaceae plants that include cruciferous vegetables such as cabbage, broccoli, cauliflower, kale, etc. are some of the most popular vegetables consumed the world over and considered to be a good source of bioactive phytochemicals. Sulfur-containing glucosides – glucosinolates (GSLs) – are one of the most important phytochemicals of *Brassica* vegetables responsible for their characteristic flavor and odor (Fahey et al., 2001; Martínez-Ballesta et al., 2013). Structurally, they are anions made up of thiohydroximates containing an S-linked β-glucopyranosyl residue and an O-linked sulfate residue with a variable amino acid-derived side chain. Unlike the other major classes of natural plant products, glucosinolates comprise a relatively small but diverse group of secondary metabolites that are generally limited to the species of the order Brassicales. These compounds represent a large chemical family that includes over 130 different compounds with varying structural subgroups (Clarke, 2010). They are largely responsible for the nutraceutical and pharmacological value of *Brassica* vegetables and have been implicated in defense against insects and pathogens and thus possess a bio-protective role. Efforts to improve specific quality attributes of plant foods, for example GSL, through breeding for quantitative food processing traits, are both promising and challenging (Hennig et al., 2013; Banerjee et al., 2014).

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The level of glucosinolate metabolites has been shown to be induced upon root colonization by *Trichoderma* (Brotman et al., 2013). Following tissue damage, GSLs are hydrolyzed *in vivo* by endogenous enzymes, myrosinase, to unstable aglycones that further rearrange to a variety of products including isothiocyanates, thiocyanates, and nitriles, the nature of which depends upon the condition of hydrolysis and the structure of the GSLs (Halkier and Gershenzon, 2006). GSLs and their hydrolytic products have been documented to have significant antimicrobial and insecticidal activities (Aires et al., 2009). These compounds are known to accumulate in *Brassica* tissues after infestation by various pathogens restricting either the spread of fungal infection or inhibiting subsequent infections. Profound (and species-specific) temporal changes in GSLs have also been reported during insect herbivory (Yan and Chen, 2007). Glucosinolate structure and levels have been shown to influence host plant suitability for generalist and specialist herbivore and their levels were altered in response to herbivory (Halkier and Gershenzon, 2006).

The concentration and type of GSLs and their hydrolysis in plants has been shown to be regulated by genetic fluctuation, by environmental factors as well as by developmental cues (Martínez-Ballesta et al., 2013). Changes in total as well as different GSL subgroups have been reported in broccoli as a result of changes in salinity suggesting a role for these compounds in the leaf water response (Martínez-Ballesta et al., 2013). Induction of GSLs in broccoli mediated by ultraviolet-B (UV-B) was found to be associated with up-regulation of genes responsive to fungal and bacterial pathogens, thus demonstrating their role as stress alleviators (Mewis et al., 2012a). These compounds are induced in response to plant signaling molecules such as SA, JA, and methyl jasmonate (MeJA), the nature and extent of elicitation being dependent on the type of elicitors (Yan and Chen, 2007). Levels of these compounds are reported to be effected under temperature and heavy metal stress and also by post-harvest storage conditions (Yan and Chen, 2007). Thus, while selenium was found to affect the content of glucosinolates in a concentration-dependent manner, cadmium stress produced no change in glucosinolate production in *B. rapa* (Kim and Juvic, 2011; Jakovljević et al., 2013). Glucosinolate concentration also increased as a result of temperature stress showing seasonal variation in *Brassica* plants (Martínez-Ballesta et al., 2013).

Van Dam et al. (2009) summarized information on the GSL levels in both root and shoot in the same plant. The authors analyzed constitutive root and shoot glucosinolates of 29 plant species, which showed that roots have higher concentrations and a greater diversity of glucosinolates than shoots. Roots have significantly higher levels of the aromatic 2-phenylethyl glucosinolate, possibly related to the greater effectiveness and toxicity of its hydrolysis products in soil. It was also seen that in shoots, the most dominant indole glucosinolate is indol-3-ylglucosinolate, whereas in roots, its methoxy derivatives are dominated. The regulation of GSL metabolism at different levels and the diverse physiological function of their metabolites indicate a complex metabolic network. Studies at the genetic level have shown that GSL metabolism interacts with cellular signaling and metabolic pathways and is regulated at different levels. The extent of GSL hydrolysis that effects interaction with microbes and herbivores is also known to be controlled at the genetic level (Sønderby et al., 2010). There is, however, a lack of understanding at the molecular level on the functional aspects such as signaling transduction pathways, control at transcriptional, translational and post-translational levels, subcellular compartmentation, and interaction with many other metabolic pathways. Further studies are needed to understand the sophisticated signaling network that connects environmental factors with GSL metabolism. Knowledge on these and related aspects can aid in metabolic engineering of *Brassica* crops for better quality, nutrition, and disease resistance.

Considerable interest in optimizing GSL content and composition for plant protection and human health has made GSLs a dynamic area in plant metabolomic research.

12.2 Glucosinolate structure, isolation, and analysis

The first general structure of GSL was proposed in 1897 by Gadamer (Fahey et al., 2001), which proposed that the side chain is linked to the nitrogen rather than to the carbon atom (Figure 12.1). Several approaches have been proposed for classification of GSLs into subgroups. The most common approach is based on the nature of the biosynthetic precursor amino acids. Another approach classifies them into aliphatic, aromatic, and indolic, or aliphatic, benzenic, and indolic derivatives. They are also grouped based on their tendency for forming specific breakdown products. Specific nomenclature of individual GSLs is generally based on naming the entire anionic structure (the central carbon (C) as well as the connected, substituted S and N) as glucosinolate and adding the systematic name of the side chain as a radical.

Glucosinolates are known to be regulated both developmentally and environmentally in various organs and tissues depending on the type of biotic and abiotic stresses. Depending on the developmental stage, tissue, and photoperiod, the distribution pattern of GSLs differs between species and ecotypes as well as between and within individual plants (Table 12.1; Martínez-Ballesta et al., 2013). Tissue-level glucosinolate accumulation has a major influence on its hydrolysis to bioactive products. Vascular tissue has been found to be the site of glucosinolate biosynthesis with endoplasmic reticulum as the subcellular location of GSL biosynthetic enzymes. Plants containing GSLs always possess a thioglucosidase called myrosinase that catalyzes the degradation of GSL substrates when plant tissue is disrupted as a result of wounding or insect and pathogen attack (Sønderby et al., 2010). The majority of the myrosinase enzymes act on multiple GSL substrates, although some of them have high specificity. Myrosinases are localized in specific cells named myrosin cells or myrosin idioblasts that contain protein-rich vacuolar-type structures termed myrosin grains. Glucosinolates, however, have been reported to be localized in vacuoles in non-specific cells together with ascorbic acid, which modulates myrosinase activity.

Substantial degradation of GSLs by myrosinase enzymes when extracted with cold organic solvent necessitates the use of hot aqueous alcohols such as methanol:water (70:30) for their isolation from plant materials (Clarke, 2010). This process denatures the enzyme and prevents hydrolysis of these compounds thereby facilitating their quantitative isolation. A prior separation into groups normally precedes their identification and quantification by instrumental methods. The presence of

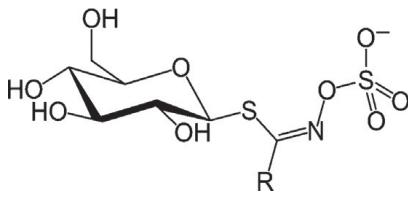

FIGURE 12.1

Structure of glucosinolate.

Table 12.1 Glucosinolates Found in Different Food Sources

Trivial Name	R Side Chain	Food Source
Glucocapparin	Methyl	Capers
Glucolepidin	Ethyl	Radish
–	Propyl	Cabbage
Glucoputranjivin	Isopropyl	Radish
Sinigrin	2-Propenyl	Cabbage
Glucoiberin	3-Methylsulfinylpropyl	Cabbage
Glucoibervirin	3-Methylthiopropyl	Cabbage
Glucocheirolin	3-Methylsulfonylpropyl	Cow's milk
Glucocapparisflexuosain	Butyl	Cabbage
Gluconapin	3-Butenyl	Cabbage
Progoitrin	(2R)-2-Hydroxy-3-butenyl	Cabbage
Epiprogoitrin	(2S)-2-Hydroxy-3-butenyl	Sea kale
Glucoerucin	4-Methylthiobutyl	Cabbage
Glucoraphanin	4-Methylsulfinylbutyl	Broccoli
Glucoerysolin	4-Methylsulfonylbutyl	Cabbage
Dehydroerucin	4-Methylthiobut-3-enyl	Daikon's radish
Glucoraphenin	4-Methylsulfinylbut-3-enyl	Radish
Glucobrassicanapin	4-Pentenyl	Chinese cabbage
Glucoberteroin	5-Methylthiopentyl	Cabbage
Glucoalyssin	5-Methylsulfinylpentyl	Rocket
Gluconapoleiferin	2-Hydroxy-pent-4-enyl	Swede
Glucosiberin	7-Methylsulfinylheptyl	Watercress
Glucohirsutin	8-Methylsulfinyloctyl	Watercress
4-Hydroxyglucobrassicin	4-Hydroxy-3-indolylmethyl	Cabbage
Glucobrassicin	3-Indolylmethyl	Cabbage
4-Methoxyglucobrassicin	4-Methoxy-3-indolylmethyl	Cabbage
Neoglucobrassicin	N-Methoxy-3-indolylmethyl	Cabbage
Glucotropaeolin	Benzyl	Cabbage
Glucosinalbin	p-Hydroxybenzyl	Mustard
Gluconasturtiin	2-Phenylethyl	Cabbage
Glucobarbarin	(2S)-2-Hydroxy-2-phenylethyl	Land cress
Glucosibarin	(2R)-2-Hydroxy-2-phenylethyl	White mustard

sulfate groups facilitates binding of these compounds to an anion exchange column and thus allows separation of either the intact GSLs or "desulfo" derivatives after enzymatic desulfation (Clarke, 2010). Direct analysis of volatile isothiocyanates and nitriles produced from GSLs by gas chromatography-mass spectrometry (GC-MS) can also provide proof of the presence of corresponding GSL in intact plants. Use of high-pressure liquid chromatography-mass spectrometry (HPLC-MSn) for detection of intact GSLs in crude extracts is currently a powerful routine method

comparable in specificity with the classical methods of identification (Clarke, 2010). Capillary electrophoresis for simultaneous quantification of GSLs and their hydrolysis products has also been reported (Clarke, 2010). Use of modern MSn equipment with ion traps allows for highly sophisticated analysis of side chain structures and validation of elucidated GSL structures. Even with highly sophisticated MS-based detection methods, comparison of chromatographic retention time with authentic standard and one additional characteristic property such as retention time in a different chromatographic system, a characteristic UV spectrum, a mass spectrum, or nuclear magnetic resonance (NMR) data is a must to suggest a tentative identification of a given GSL.

12.3 Biosynthesis of glucosinolates

Biosynthesis of GSLs involves three independent stages, namely: (1) chain elongation of selected precursor amino acids (mainly methionine) by addition of methylene groups; (2) formation of core glucosinolate structure by reconfiguration of the amino acid moiety; and (3) secondary modification of the amino acid side chain by hydroxylations, methylations, oxidations, or desaturations. While the construction of core anionic structure from amino acids involves a number of common steps, a number of diverse steps are involved in formation of side chain and other diversifications. Aliphatic GSLs are derived from alanine, leucine, isoleucine, valine, and methionine, while benzenic GSLs are formed from phenylalanine and tryptophan and indolic GSLs from tryptophan (Sønderby et al., 2010).

Synthesis of the core GSL structure is achieved in five steps (Figure 12.2). The first step involves oxidation of precursor amino acids to aldoximes by side chain-specific cytochrome P450 monooxygenase of the CYP79 family. Further oxidation by cytochrome P450 of the CYP83 family leads to aci-nitro compounds or nitrile oxides. The nitro compounds formed are strong

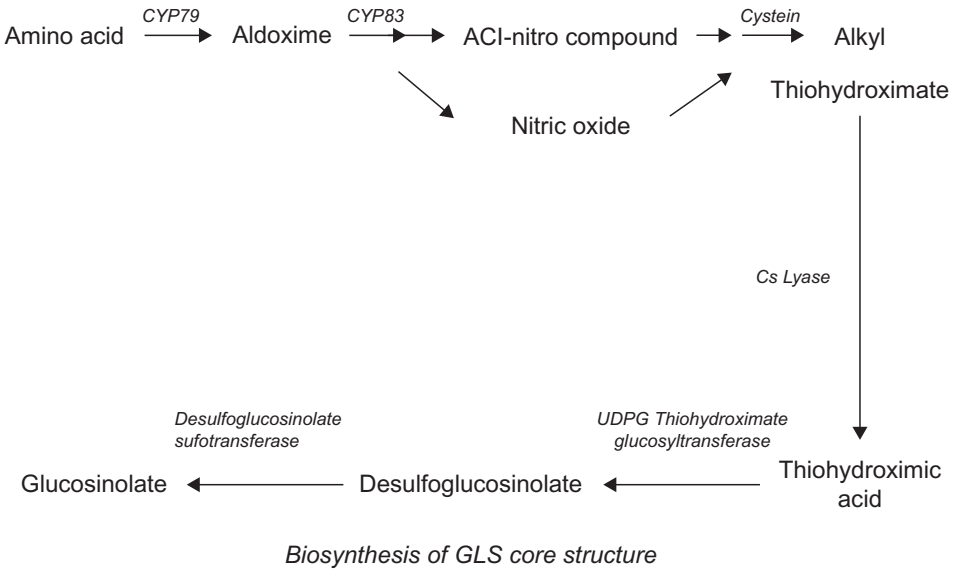

FIGURE 12.2

Biosynthesis of glucosinolate core structure.

electrophiles that react spontaneously with thiols to form S-alkylthiohydroximate conjugates that further undergo cleavage into unstable thiohydroximates, pyruvate, and ammonia by the action of a C−S lyase. Glucosyl transferase catalyses thiohydroximate-specific S-glycosylation. The final step is the 3′-phosphoadenosine 5′-phosphosulfate-dependent sulfation of desulfoglucosinolates (Sønderby et al., 2010).

The amino acid elongation is similar to the valine-to-leucine conversion and involves five steps which include initial and final transamination, acetyl-CoA condensation, isomerization, and oxidative decarboxylation. Methylthioalkylmalate (MAM) synthases that catalyze the condensation reaction have been characterized in *Arabidopsis* and *Eruca sativa*. Methionine side chain elongation occurs in the chloroplast and elongated α-keto acid can either be transaminated and enter the core GSL pathway or undergo additional elongation steps with insertion of up to nine methylene units. The variation in side chain length of methionine-derived GSL is controlled by three partially redundant MAM genes (Sønderby et al., 2010).

Secondary modification of the side chains involving various types of oxidations, eliminations, alkylations, and esterifications is generally considered as the final stage in GSL synthesis. An extensive natural variation of aliphatic glucosinolates has been noted in *Arabidopsis* with two α-ketogluterate-dependent dioxygenases controlling the production of alkenyl and hydroxyalkyl GSLs (Kliebenstein et al., 2001).

An interdependent metabolic control of aliphatic and indolyl GSL branches has been proposed indicating a homeostatic control of GSL synthesis. This is achieved by a reciprocal negative feedback regulation between both the branches using intermediates or end products of glucosinolate biosynthesis as inhibitors. Limited NADPH supply has also been proposed for the interdependence of the two pathways wherein inhibition of one branch would lead to increased NADPH availability for the other. In addition, side chain elongation can lead to extra yield of NADH that can be converted to NADPH via the malate dehydrogenase and maleic enzyme reactions. Thus, side chain elongations can provide NADPH independently of the pentose-phosphate pathway thereby increasing GSL production. Considerable variation is thus noted in the total as well as individual GSL content of methionine-derived and indolyl GSL in leaves and seeds, respectively (Grubb and Abel, 2006).

12.4 Role of glucosinolates in stress alleviation

Loss of cellular integrity as a consequence of stress induced by wounding, insect, or pathogen attack leads to hydrolysis of GSLs by the enzyme myrosinase. GSLs and their hydrolytic products are frequently investigated for their role as a plant defense system against insects, herbivores, and certain microbial pathogens. It has been shown that infection with fungal pathogen can induce local synthesis of myrosinase and the possibility of such a mechanism under other stress response is also proposed. Environmental factors influence secondary metabolism as plants under stress produce more secondary metabolites, more so as the growth is often limited more than in photosynthesis, and carbon fixation is predominantly invested to secondary metabolite production (Endara and Coley, 2011). It has been very well reported that environmental factors, such as light (Engelen-Eigles et al., 2006), temperature (Velasco et al., 2007), salinity (Qasim et al., 2003; López-Berenguer et al., 2009), water (Champolivier and Merrien, 1996; Rask et al., 2000), CO_2 (Schonhof et al., 2007a), and drought (Radovich et al., 2005) may affect glucosinolate levels (Table 12.2).

Table 12.2 Impact of Abiotic Stress on Glucosinolate Accumulation in Different *Brassica* Plant Species

Plant Species	Glucosinolate Content	Stress	Treatment Condition
Brassica oleracea L. var. *italic*	Increase	Salinity	NaCl (40, 80 mM), during 2 weeks
Brassica rapa L.	Increase		NaCl (20, 40, 60 mM), during 5 days
Brassica campestris L. ssp. *chinensis* var. *communis*			NaCl (50 and 100 mM for 2 weeks)
Brassica oleracea L. var. *capitata*	Increase	Drought	Severe stress 2 weeks
Brassica oleracea L. var. *italica*	Increase		Severe stress 2 weeks
Brassica napus L.	Increase		Severe stress more than 1 week
Brassica rapa ssp. *rapifera* L.	Increase		Mild stress—25% of available water
Brassica carinata L.	Increase/no effect		Mild and severe stress (40, 23, 17 and 15% of available water)
Brassica oleracea L. var. *gemmifera*	No effect		Mild stress (30% of available water)
Brassica napus L.	No effect		Mild stress
Brassica oleracea L.	Decrease		Mild and severe stress (40−45% of available water)
Arabidopsis thaliana L.	Decrease		Severe stress
Arabidopsis thaliana (L.)	Decrease		Mild stress (50% of available water)
Arabidopsis thaliana (L.)	Decrease		Water logging (200% of available water)
Brassica rapa L.	Increase	Temperature	Elevated temperature (21−34°C)
Brassica rapa L.	Decrease		Low−medium temperature (15−27°C)
Brassica oleracea L.	Increase		Elevated temperature (32°C)
Brassica oleracea L.	Decrease during day/ increase during night	Light cycling	14 h/10 h day/night*
Arabidopsis thaliana L.	Increase upon light/ decrease upon darkness		16 h/8 h d/n or continuous darkness
Brassica oleracea L. var. *italica*	Increase upon light		16 h/8 h d/n or continuous darkness
Arabidopsis thaliana	Slight increase	UV-B radiation	1.55 Wm^{-2}
Brassica oleracea L. var. *italica*	Increase		Up to 0.9 kJm^{-2} d^{-1}
Brassica oleracea L. var. *italica*	Increase	Nutrient availability	N-limitation (1 gr N pot^{-1})

(*Continued*)

Table 12.2 (Continued)

Plant Species	Glucosinolate Content	Stress	Treatment Condition
Brassica rapa ssp. *rapifera* L	Increase		S-supply (60 kg S ha^{-1})
Brassica oleracea L. var. *italica*	No effect		S-supply (150 kg/ha)
Brassica oleracea L. *capitata*	Increase		S-supply (110 kg S ha^{-1})
Brassica napus	Increase		S-supply (100 kg S ha^{-1})
Tropaeolum majus	Increase		S-supply (8.3 mM SO_4^{2-})
Brassica oleracea L. var. *italica*	No effect		S-limitation (15 kg/ha)
Arabidopsis thaliana L.	Increase		K-deficiency (lack KNO_3 for 2 weeks)
Brassica rapa L.	Decrease		K-deficiency (lack of nutrient solution for 5 days)
Brassica oleracea L. var. *italica*	Increase		Se-supply (5.2 mM Na_2 SeO_4)
Brassica oleracea L. var. *italica*			B-deficiency (9–12 μg gr DW^{-1})
Cabbage and kale	Increase	Cadmium	Cd (5 and 10 mg Cd kg^{-1} soil)
Thlaspi caerulescens	Increase		

Source: Modified after Martínez-Ballesta et al., 2013.

As *Brassica* crops contain high amounts of sulfur-containing amino acids and glucosinolates, glucosinolate metabolism and the effects of sulfur and nitrogen nutrition have been studied (Schnug et al., 1993; Krumbein et al., 2002; Salac et al., 2006; Schonhof et al., 2007b). It is evident that when broccoli plants were supplied with low sulfur or nitrogen, a decrease in glucosinolates was noted, whereas total glucosinolate levels were elevated at sufficient nitrogen supply or high sulfur levels, and were lower at low sulfur supply with an optimal nitrogen supply (Aires et al., 2006; Schonhof et al., 2007a). Similarly, glucosinolate levels in turnip were found to be strongly regulated by nitrogen and sulfur application (Kim et al., 2002). In field experiments, nitrogen and sulfur supply showed a clear influence on individual glucosinolates as it may favor the hydroxylation step converting but-3-enyl glucosinolate to 2-hydroxybut-3-enyl glucosinolate. Compared to indole glucosinolates, aliphatic glucosinolates show a greater sensitivity to sulfur deficiency probably because they are synthesized from methionine (Zhao et al., 1994). Some *B. napus* cultivars with reduced contents of aliphatic glucosinolates were more sensitive to sulfur deficiency (Schnug, 1990), which suggests a role of aliphatic glucosinolates in the survival strategy to mineral stress. Sulfur fertilisation leads to increases in glucosinolate content in most cases. Increases of over 10-fold have sometimes been reported. For example, the benzyl glucosinolate content of *Tropaeolum majus* was increased over 50-fold by fertilising a particular cultivar with 8.3mM sulfate (Matallana et al., 2006).

12.4.1 Biotic stress

During their lifetime, plants have to deal with a variety of environmental stresses including biotic stresses such as those from microbial pathogens and herbivores. As plants are not in a position to move from their unfavorable environment, they have evolved a broad range of defense mechanisms. The role of GSLs in combating biotic stress has been well recognized. GSLs exhibit growth inhibition or feeding deterrence to a wide range of general herbivores such as birds, slugs, and generalist insects (Rask et al., 2000; Barth and Jander, 2006). Plants respond to herbivore or insect damage by accumulating higher GSL levels and thus increase their resistance to such biotic stresses. Glucosinolates, the characteristic secondary compounds of Brassicaceae, as well as proteinase inhibitors, remained unaffected by UV in all plants, demonstrating independent regulation pathways for different metabolites (Kuhlmann and Müller, 2009a,b). Mewis et al. (2012b), however, demonstrated an increase in aliphatic GSLs in *Arabidopsis thaliana* when fed by phloem-feeding aphids, the green peach aphid (*Myzus persicae*), cabbage aphid (*Brevicoryne brassicae*), and generalist caterpillar species *Spodoptera exigua.* Interestingly, the content of indole GSLs were found to be unchanged. GSL levels have been demonstrated to reduce damage by generalist herbivores. Volatiles produced by GSLs can also provide indirect protection to plants by attracting natural enemies of herbivores such as parasitoids. Several reports exist on the toxicity of GSL hydrolysis products to bacteria and fungi (Mayton et al., 1996; Brader et al., 2001). Pedras and Sorensen (1998) demonstrated an inhibitory action by various isothiocyanates derived from GSLs on germination and growth of a fungal pathogen *Leptosphaeria maculans*. Aromatic isothiocyanates were found to be more toxic than aliphatic isothiocyanates and the fungal toxicity of the latter decreased with increase in side chain length. In a study on the antimicrobial effect of crude extracts from *Arabidopsis*, Tierens et al. (2001) identified 4-methylsulfonyl butyl isothiocyanate as the major active compound with a broad spectrum of antimicrobial activity. Thus, the possible protective role of GSL-derived isothiocyanate against pathogens was demonstrated. Investigation of the level of GSLs in different *Brassica* cultivars by several workers indicated changes in GSL pattern when inoculated by fungal pathogens. These changes were mostly due to increase of indole and aromatic GSLs, although increase of aliphatic GSLs was also noted.

12.4.2 Abiotic stress

All abiotic stresses are important environmental factors that reduce plant growth and yield. Plants respond and adapt to these stresses in order to survive. Signaling pathways are induced in response to environmental stresses. Several signaling molecules have been identified in plant defense responses. These include JA, SA, and ET, which have been demonstrated to operate independently and/or synergistically in different signal transduction pathways. JA and SA have been shown to be involved in the induction of different GSLs (Yan and Chen, 2007). Different signal transduction pathways activate specific biosynthetic and secondary modifying enzymes, leading to altered levels of specific GSLs. The induction of GSLs by several defense pathways strongly indicates that these compounds play a role in plant defense.

Salt stress is a major abiotic stress reducing the productivity of crops in many areas of the world. Salinity affects the water balance resulting in osmotic damage. Osmotic adjustment is a

plant adaptation mechanism used to maintain water balance in plants. In their studies on the effect of salinity stress on GSL content, Keling and Zhujun (2010) found a considerable influence of NaCl stress on the GSL content and composition in pakchoi (*Brassica campestris* L. ssp. *chinensis* var. *communis*) shoots. At 50 mM NaCl, the contents of total GSLs as well as aliphatic and indole GSL significantly increased. A significant increase in indole GSLs and a decrease in aromatic GSL (gluconasturtiin) were, however, noted at 100 mM NaCl. Glucoalyssin, gluconapin, glucobrassicin, and neglucobrassicin were significantly enhanced at 50 mM NaCl, while only the content of gluconapin and glucobrassicin increased at 100 mM NaCl.

Drought stress resulted in considerably elevated leaf GSL content of *Brassica carinata* varieties with the magnitude of GSL concentration varying with the stage of development and intensity of the drought stress (Schreiner et al., 2009a). Increase in leaf GSL concentrations correlated with relative water content with reduced water content leading to higher leaf GSL concentration. *Brassica oleracea* L., plants grown for two weeks under drought stress showed decreased levels of indolyl GS when compared to well-watered plants, while water logging conditions resulted in slight increases within the GS contents (Khan et al., 2011). Imbalance in sulfur to nitrogen ratio may result in the alteration of nutrient uptake due to water deficit resulting in the accumulation of GSLs as sulfur sink. Further, stresses such as low water availability change the hormonal distribution of plants leading to a cascade of signal transduction pathways that result in the expression of stress-responsive genes. Particularly, stress hormones like ABA, JA, ethylene, and SA that play a very important role in biotic and abiotic stress resistance are known to increase the concentrations of GSLs (Yan and Chen, 2007).

UV-B radiation acts as an environmental stress and triggers various responses in plants. These include changes in growth, development, morphology, and physiological aspects. In recent years, some researchers have reported the effect of UV-B on GSL metabolism. Microarray data have shown that the genes related to the biosynthesis of flavonoids, glucosinolates, and terpenoids were differently expressed after UV-B radiation. A study on the effect of UV radiation on *Tropaeolum majus* demonstrated that appropriate UV-B dosage could increase the glucotropaeolin concentration (Schreiner et al., 2009b). Wang et al. (2011) showed that UV-B radiation induced the production of GSLs. Continuous UV-B exposure for 12 h, however, inhibited the expression of glucosinolate metabolism-related genes resulting in a significant decline in glucosinolate content, particularly that of indolic glucosinolates. In another study on UV-B-mediated induction of GSLs, Mewis et al. (2012c) reported the induction of of 4-methylsulfinyl butyl GSL and 4-methoxy-indol-3-ylmethyl GSL in sprouts of *Brassica oleracea* var. *italica* (broccoli). Accumulation of defensive GSLs was accompanied by increased expression of genes associated with salicylate and JA signaling defense pathways and up-regulation of genes responsive to fungal and bacterial pathogens. Enhanced GSL formation had a negative effect on the growth of aphid *Myzuz persicae* and attack by caterpillar *Pieris brassicae*. Levels of these compounds are also reported to be effected under temperature stress. The TU8 mutant of *Arabidopsis* deficient in glucosinolate metabolism and pathogen-induced auxin accumulation showed less tolerance to elevated temperatures than wild-type plants (Ludwig-Müller et al., 2000). Seasonal variation in the concentration of aliphatic, aromatic, and indole GSLs was noted in different varieties of *Brassica oleraceae* (Cartea et al., 2008). Similar effects with increase in aliphatic glucosinolates (particularly glucoraphanin) were observed in broccoli kept at daily mean temperatures between 7 and 13°C (mean radiation of 10−13 mol m^{-2} day^{-1}) (Schonhof et al., 2007c).

In the authors' laboratory (Banerjee et al., 2014), the cabbage leaves subjected to gamma radiation stress were found to have an enhanced sinigrin content. No effect of myrosinase activity was, however,

noted, thus providing high retention of glucosinolates and facilitating improved release of these nutraceutically significant compounds during mastication of the vegetable. Thus, exposure to such abiotic stress was demonstrated to provide improved benefit in terms of enhancing intake of potentially important health protective and promoting compounds in *Brassica* vegetables (Banerjee et al., 2014).

Heavy metal stress also can lead to changes in GSL content. While selenium was found to affect the content of GSLs in a concentration-dependent manner, cadmium stress produced no change in GSL production in *B. rapa* (Kim and Juvic, 2011; Jakovljević et al., 2013). GSL concentration also increased as a result of temperature stress showing seasonal variation in *Brassica* plants. In *Thlaspi caerulescens*, a metal hyperaccumulator with a high requirement of zinc, GSL levels (particularly sinalbin) increased in roots but decreased in leaves and shoots. Zinc had a clearly distinctive effect on the specific group of indolyl GSLs in *T. caerulescens* with a drastic reduction in both roots. Post-harvest storage conditions of *Brassica* vegetables are also known to influence GSL and related isothiocyanate content. Content of these compounds was found to decrease in vegetables such as broccoli, brussel sprouts, cauliflower, and green cabbage when stored in a domestic refrigerator (4−8°C) for 7 days unlike when stored at ambient temperature (Song and Thornalley, 2007). Storage of vegetables at very low temperature (−85°C) can result in freeze−thaw fracture of plant cells leading to significant loss of GSLs as a consequence of their conversion to isothiocyanates during thawing (Song and Thornalley, 2007). Tamara et al. (2013) found that GSLs in leaves and root could be more involved in ameliorating S deficiency rather than plant defense in the short term in cadmium (Cd) stress; however, total GSL levels in the stem during the long term could serve as a GSL storage organ implying possible roles of GSL in Cd stress.

12.5 Genes involved in glucosinolate biosynthesis

The main genetic pathway of glucosinolate biosynthesis has been identified in *Arabidopsis* using genetic and biochemical approaches. Several enzymes and transcription factors involved in the GSL biosynthesis have been studied in the model plant, *Arabidopsis*, and in a few other *Brassica* crop species (Baskar et al., 2012). Figure 12.3 presents the genetic machinery involved in different aspects of GSL sysnthesis. Six MYB factors, namely, MYB28, MYB29, MYB76, MYB34, MYB51, and MYB122, have been found to be transcriptional regulators in the biosynthesis of glucosinolate in *Arabidopsis*. While MYB28, MYB29, and MYB76 specifically transactivate genes related to aliphatic glucosinolate biosynthetic pathway (MAM3, CYP79F1, and CYP83A1) (Gigolashvili et al., 2007b, 2008), MYB34, MYB51, and MYB122 are regulators of the indolic glucosinolate biosynthetic pathway (TSB1, CYP79B2, and CYP79B3) (Celenza et al., 2005; Gigolashvili et al., 2007a). Wang et al. (2011) used the comparative genomic analysis method of *Arabidopsis thaliana* and *Brassica rapa* and identified 102 putative genes in *B. rapa* as the orthologues of 52 *Arabidopsis* glucosinolate genes. The glucosinolate genes in *B. rapa* and *A. thaliana* shared 59−91% nucleotide sequence identity. Microarray experiments have also shown that CYP79B2, an important gene involved in the biosynthesis of indolic glucosinolates (Chen and Andreasson, 2001), is downregulated by brassinosteroids (Goda et al., 2002). Both MYB34 and MYB51, which encode transcriptional factors of indolic glucosinolate biosynthesis, contain a BZR1 binding site in their promoters (Sun et al., 2010). Further, Guo et al. (2012) investigated the role of

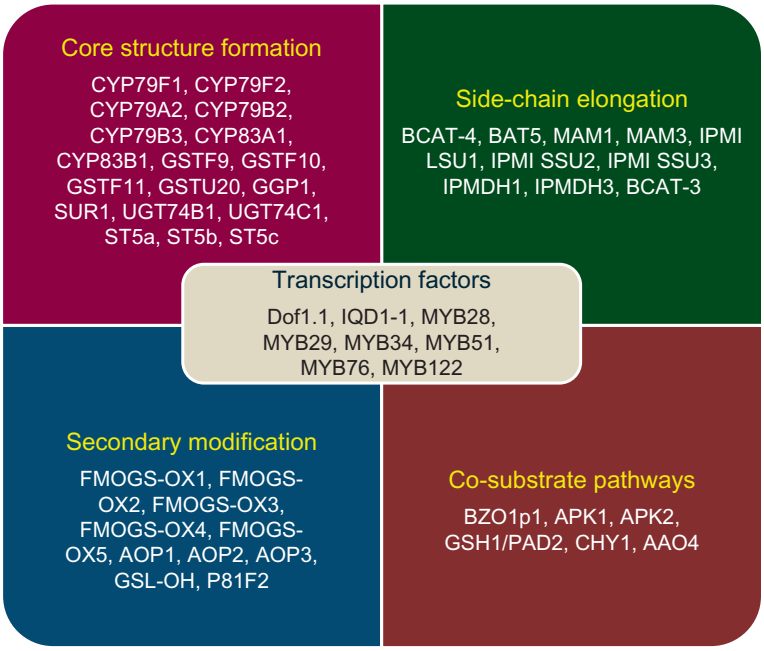

FIGURE 12.3

Genes involved in different stages of glucosinolate biosynthetic pathway.

brassinosteroids in glucosinolate biosynthesis in *Arabidopsis* using mutants and transgenic plants. Zang et al. (2009) identified glucosinolate synthesis genes in *Brassica rapa* on the basis of cDNA and BAC libraries with about 21.5% of the genes as partial CDS sequences and many BrGS genes anchored only on the BAC, rather than on chromosomes. The authors also identified glucosinolate biosynthetic genes by comparative genomic analysis between *B. rapa* and *A. thaliana*. Augustine et al. (2013) analyzed four MYB28 genes that are differentially expressed and regulated in both a tissue- and cell-specific manner in controlling aliphatic glucosinolate biosynthesis in *B. juncea.*

Several myrosinase genes from *Sinapis alba*, *Brassica napus*, and *Arabidopsis thaliana* have been isolated and characterized indicating that myrosinases are encoded by a multigene family consisting of three subgroups (Xu et al., 2004). Myrosinase in the *Brassica* family is encoded by a gene family, which consists of three subfamilies, namely, MA (Myr1), MB (Myr2), and MC (Baskar et al., 2012). Several myrosinase-associated proteins, such as epithiospecifier modifier 1 (ESM1), ESP, and MVP1, have been identified in *Arabidopsis*, which are mainly involved in the generation of diversified GSL metabolic products (Baskar et al., 2012).

12.6 Gene expression profiling in response to environmental cues

Plant glucosinolate metabolism is responsive to many environmental factors. Generally, glucosinolate degradation products serve as defense compounds against pathogens and generalist herbivores, and as attractants to glucosinolate-adapted specialists (Rask et al., 2000; Barth and Jander, 2006).

Several glucosinolate hydrolysis products have been reported to display toxicity to fungi and bacteria (Mayton et al., 1996; Brader et al., 2001). Glucosinolate levels in oilseed rape were positively correlated with resistance to pathogens (Li et al., 1999) with some exceptions (Giamoustaris and Mithen, 1997). The best *in vivo* evidence for the defense role of glucosinolates came from an *MAM1* mutant study, where decreased glucosinolate levels in *Arabidopsis* caused susceptibility to *Fusarium oxysporum* (Tierens et al., 2001). Pathogen infection can also change glucosinolate profiles. When *Brassica* plants were infected by *Leptosphaeria maculans*, glucosinolate levels were induced, but myrosinase levels were not affected (Siemens and Mitchell-Olds, 1998). JA and SA signaling pathways may be involved in the regulation of glucosinolate levels (Li et al., 2006). Currently, there is more literature on plant interactions with insect herbivores. When glucosinolate levels increased in *B. napus* and *Sinapis alba*, feeding by generalist insects decreased significantly, while feeding by specialist insects (e.g., *Pieris rapae*) greatly increased and caused severe damage (Giamoustaris and Mithen, 1995). The damage led to a systemic increase in indole glucosinolate and often in total glucosinolate levels. For example, when seedlings of oilseed rape and mustard were attacked by *Xea* beetles, there was a tremendous increase in the concentration of indole glucosinolates, but no significant changes in aliphatic glucosinolates (Bodnaryk, 1992; Bartlet et al., 1999). In one case when feeding with turnip root fly, the concentrations of aliphatic glucosinolates actually dropped (Hopkins et al., 1998).

Mewis et al. (2006) analyzed glucosinolate accumulation levels and gene expression of glucosinolate biosynthetic genes in response to feeding by four herbivores in *Arabidopsis thaliana* (L.) wild-type (Columbia) and mutant lines that were affected in defense signaling. Herbivory on wild-type plants led to increased aliphatic glucosinolate content for three of four herbivores tested, namely, *Myzus perscae* (Sulzer), *Brevicoryne brassicae* (L.), and *Spodoptera exigua* Hübner. The lepidopteran *Pieris rapae* L. did not affect the levels of aliphatic glucosinolate in the wild type, except for an increase in indole glucosinolates. Increased expression of genes of aliphatic glucosinolate biosynthesis was observed after feeding by all species, while mutations in jasmonate (*coi1*), salicylate (*npr1*), and ethylene signaling process (*etr1*) showed varied gene expression, glucosinolate profile, and insect performance compared to wild type. As against in wild type, the gene transcripts of aliphatic glucosinolate biosynthesis did not generally increase in the mutants. Both glucosinolate content and gene expression data indicated that salicylate and ethylene signaling repress some jasmonate-mediated responses to herbivory. This possibly indicates that all three modes of signaling processes are involved in responses to herbivores.

Plant interactions with the environment influences glucosinolate metabolism and is constantly regulated by different environmental factors including UV-B radiation. Wang et al. (2011) studied the glucosinolate content and expression of glucosinolate metabolism-related genes in response to enhanced UV-B radiation (1.55 $W \bullet m^{-2}$) and the succeeding dark recovery process in *Arabidopsis thaliana* rosette leaves. Induction of glucosinolates was observed in the 1 h of enhanced UV-B radiation exposure, whereas, after continuous exposure for 12 h, the expression of glucosinolate metabolism-related genes was significantly inhibited and the glucosinolate content was declined, especially that of indolic glucosinolates. Upon exposure to darkness for 12 h for partially recovery, both glucosinolate gene expression and the content returned to the control levels. The results of Wang et al. (2011) showed up-regulation of some genes (MYB51, OBP2, MYB76, SOT16, and TGG1) in the initial 1 h of UV-B exposure. These genes were also induced by JA and wounding (Skirycz et al., 2006; Dombrecht et al., 2007; Staswick, 2008). The transcription factors (MYB51,

OBP2, and MYB76) can positively mediate glucosinolate biosynthesis (Skirycz et al., 2006; Dombrecht et al., 2007; Gigolashvili et al., 2008). MYB51 and MYB76 specifically activate indolic and aliphatic glucosinolate biosynthesis, respectively, which might be responsible for the temporary and initiative increase of 4MSOB and I3M. The 3 h of treatment led to down-regulation of MYB28 and MYB29 (the genes encoding the transcription factors that regulate aliphatic glucosinolate biosynthesis) and MYB34 (regulating indolic glucosinolate biosynthesis), and structural genes (CYP79F1, CYP83B1, MAM1, CYP79B2, and CYP79B3) encoding enzymes catalyze the synthesis of aliphatic glucosinolates. Subsequent exposure led to decline in the expression of the majority of genes and the glucosinolate contents. Microarray analysis experiments with UV-B exposed *Tropaeolum majus* also indicated differential expression of genes related to the biosynthesis of flavonoids, glucosinolates, and terpenoids (Hectors et al., 2007).

Schweizer et al. (2013) showed that a triple mutant for *MYC2*, *MYC3*, and *MYC4*, three basic helix−loop−helix transcription factors that are known to additively control jasmonate-related defense responses, was shown to have a highly reduced expression of GSL biosynthesis genes. The myc2 myc3 myc4 (myc234) triple mutant was almost completely devoid of GS and was extremely susceptible to the generalist herbivore *Spodoptera littoralis*.

12.7 Signaling networks

The biotic and abiotic factors such as pathogen challenge, herbivore damage, mechanical wounding, or altered mineral nutrition are known to regulate glucosinolate profiles (Wittstock and Halkier 2002; Agrawal and Kurashige 2003; Mewis et al., 2005). Sulfur limitation is shown to repress most glucosinolate pathway genes (Hirai et al., 2005). The bioinformatics approach has enabled identification of core pathway genes and predicted additional enzymes with roles in glucosinolate biosynthesis. In addition to this modulation, plant hormones such as jasmonates, SA, and ET associated with specific and broad-spectrum defense responses can also affect glucosinolate content (Brader et al., 2001; Kliebenstein et al., 2002; Mikkelsen et al., 2003a; Mewis et al., 2005). Jasmonates known to be involved in responses to insect attack and necrotrophic pathogens have shown increased indolyl and specific aliphatic glucosinolates (Brader et al., 2001; Mikkelsen and Halkier, 2003b), possibly via multiple signaling pathways (Kliebenstein et al., 2002). These studies have demonstrated the utility of mutants defective in hormone synthesis or signaling through the regulation of specific indole glucosinolate production by SA as well as interactions of jasmonate and salicylate signaling.

NPR1 (*nonexpresser of PR genes 1*), ETR1 (*ethylene receptor 1*), and COI1 (*coronatine insensitive 1*) are important for SA, ET, and JA signaling, respectively. It is also evident that insect feeding-induced glucosinolate biosynthesis requires the functions of regulatory proteins NPR1 and ETR1, but not COI1 (Mewis et al., 2005). As the SA and JA pathways seem to be mutually antagonistic (Glazebrook et al., 2003), blocking of JA signaling or increased SA signaling caused reduction of glucosinolate levels and vice versa (Mikkelsen et al., 2003a,b; Mewis et al., 2005; Li et al., 2006). While ET signaling may influence other resistance traits than glucosinolates, NPR1 may be a point of crosstalk of multiple signaling pathways (Glazebrook et al., 2003; Mewis et al., 2005). Mewis et al. (2005) found that exogenous application of JA did not mimic plant responses to insect feeding in terms of glucosinolate metabolism. Potassium starvation was shown to induce the expression of

JA biosynthetic genes and glucosinolate metabolic genes. Resupply of potassium down-regulated the transcription of these genes (Armengaud et al., 2004). Defense responses are not only activated at the site of wounding but also distantly in other remote plant parts. Chen et al. (2013) suggested that higher contents of indole glucosinolates in systemic leaves might arise from the induction of a long-distance signal produced in local leaves as well as from JA synthesized in systemic leaves.

Studies have demonstrated that wound, pathogen, and hormones like JA and ET induced expression of transcriptional factor genes (Schenk et al., 2000) and that nuclear proteins regulate glucosinolate metabolism (Yan and Chen, 2007). Among the transcription factor genes, ATR1, a Myb transcription factor, regulates a number of genes of tryptophan biosynthesis and indole glucosinolate biosynthesis. Specifically, ATR1 participates in the JA-mediated induction of indole glucosinolate biosynthesis (Celenza et al., 2005). Transcription factors AtDof1 and Myb51 also control indole glucosinolates. While AtDof1 regulates CYP83B1 and is inducible by generalists and JA (Skirycz et al., 2006), both AtDof1 and Myb51 activate ATR1 and myrosinase binding proteins (Skirycz et al., 2006; Gigolashvili et al., 2007b). It has also been shown that overexpression of AtDof1 caused changes in aliphatic glucosinolate levels, similar to overexpression of a novel calmodulin binding nuclear protein IQD1, which led to high levels of both indole and aliphatic glucosinolates (Levy et al., 2005). The study suggested that regulation of aliphatic glucosinolate metabolism may not occur at transcriptional level and crosstalk may in fact operate between regulatory pathways of indole glucosinolates and aliphatic glucosinolates. Hirai et al. (2007) investigated Myb28 and Myb29 as master transcription factors; while Myb28 regulates the pathway from methionine to aliphatic glucosinolates, and is essential for the basal-level control of aliphatic glucosinolate biosynthesis, Myb29 has a role in JA-mediated aliphatic glucosinolate biosynthesis (Hirai et al., 2007).

The regulation of cellular processes in a cell are fine-tuned by post-translational modifications, especially protein phosphorylation and redox regulation (Paget and Buttner, 2003; Chen and Harmon, 2006). Several protein kinases, including receptor-like protein kinases, protein phosphatases, and MAP kinases, are shown to respond to pathogens, JA, and hydrogen peroxide treatments (Desikan et al., 1999; Schenk et al., 2000). Desikan et al. (2001) found that protein kinases, phosphatases, and calmodulin proteins as well as myrosinases and myrosinase binding proteins were affected by oxidative stress often impacting the accumulation of hydrogen peroxide and ROS (Apel and Hirt, 2004). Abiotic and biotic stresses cause changes in glucosinolate metabolism and hence it can be assumed that redox modifications may also play an important role in regulating glucosinolate metabolism.

12.8 Metabolic engineering of glucosinolates

Metabolic engineering of glucosinolates can be achieved by targeting either the biosynthetic or the transcription factors of the GSL biosynthetic pathway. More than 20 genes with potential regulatory function in GSL metabolism and several transcription factors have been identified in *Arabidopsis* and other plant species. Manipulation of these transcription factors appears to be more effective for the control of metabolic pathways than that of genes encoding single enzyme in plants (Capell and Christou, 2004). Further efforts in this direction will certainly provide the required insights to facilitate the modification of the complex GSL biosynthesis of plants in the near future. Baskar et al. (2012) described different strategies including overexpression of *CYP79A1*, *CYP71E1*, MAM1, *CYP79F1, CYP83A1*, etc., to produce genetically engineered plants with altered GSL profiles.

In addition to plants, microbial source has also been attempted as a great potential for large-scale production of desirable GSLs for the benefit of human health (Mikkelsen et al., 2012) through the stable expression of multigene pathways from *Arabidopsis* to yeast.

12.9 Conclusion and future prospects

Glucosinolates are a diverse group of secondary metabolites largely responsible for the nutraceutical and pharmacological value of *Brassica* vegetables and have been implicated in defense against insects and pathogens and thus possess a bio-protective role. Recent years have witnessed great progress in the understanding of glucosinolate biosynthesis in model plants like *Arabidopsis thaliana* using different biochemical and reverse genetics approaches. Cooperation between researchers involved in plant breeding and food technology could lead to using food technological parameters as breeding traits to identify genetic loci associated with food processing to breed vegetables with higher retention of glucosinolates (Hennig et al., 2013).

Abiotic stresses, such as salinity, drought, extreme temperatures, light and nutrient deprivation, modulate the glucosinolate profiles through different mechanisms through the involvement of hormones or signaling molecules. The accumulation of glucosinolates is also defined by the magnitude and duration of the stress impact, for example plant−pathogen interactions where the plant water availability and herbivore feeding or pathogen attack come into the picture. It has also been seen that exogenous glucosinolate hydrolysis products (isothiocyanates) alleviate the impact of drought or elevated temperatures. It is yet to be investigated how molecular mechanisms operate in this alleviation process, and also allocation and/or redistribution of glucosinolates in response to environmental changes. As the isothiocyanates produced from the Val- and isoleucine-derived glucosinolates are volatile, metabolically engineered plants producing these glucosinolates have novel properties with great potential for improvement of resistance to herbivorous insects and for biofumigation. Regulation and control of glucosinolate metabolism also needs to be investigated at different levels of signaling and metabolic network that control this pathway. It is also desirable to identify genes/loci in different *Brassica* species so that they might be used to manipulate aliphatic glucosinolates towards favorable forms (Li et al., 2008; Baskar et al., 2012). Understanding the dynamics of the glucosinolate biosynthesis network will not only advance our basic knowledge of this bioactive molecule complex but also augment research efforts towards metabolic engineering.

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