In vitro regeneration and adventitious root culture of *Brassica oleracea* var. *acephala*

By

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In vitro regeneration and adventitious root culture of *Brassica oleracea* var. *acephala*

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In

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By

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Supervised by

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God does not charge a soul with more than it can bear

It shall be requited for whatever good and

whatever evil it has done.

Our Lord!

Take us not to task

if we forget, or Lapse into error

our Lord!

Charge us not with the burden

You laid upon those before us.

Our Lord!

do not burden us

beyond what we have the strength to bear

and pardon us

and forgive our Sins,

and have mercy on us

You alone are our protector

and help us against people

who deny the Truth.

(Ameen)

Dedicated

to

My Parents

At this day, this moment,

What I am is because of my Parents.

If I want to thank them,

I could not, as they deserve to be thanked,

But my whole life is at their service.

DECLARATION

The whole of the experiment work including lab and field described in this thesis was carried out by me in the Plant Tissue Culture Laboratory, Department of Biotechnology, Quaid-i-Azam University Islamabad. The findings and conclusions are of my own investigation with discussion of my supervisor Dr. Bilal Haider Abbasi. No part of this work has been presented for any other degree.

MUHAMMAD ADIL

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LIST OF ABRREVIATIONS

BAP	6-Benzyl aminopurine
DPPH	1, 1-diphenyl-2-picrylhydrazyl
TDZ	Thidiazuron
GA_3	Gibberellic acid
MS0	MS medium without plant growth regulators
NAA	α-Naphthaleneacetic acid
PGRs	Plant growth regulators
2,4 D	2,4 dichlorophenoxyacetic acid
IBA	Indole butyric acid
IAA	Indole acetic acid

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Abstract

The in vitro regeneration, adventitious root culture and antioxidant activity of different regenerated tissues of Brassica oleracea var. acephala were investigated. Indirect shooting was induced from leaf explant of in vitro seed derived plants on MS medium containing different plant growth regulators. The optimum callus formation was observed on MS medium containing Naphthalene Acetic Acid (NAA, 1.0 mg/l) and 6-Benzyladenine (BA or BAP, 1.5 mg/l) or Thidiazuron (TDZ, 2.5 mg/l) + NAA (1.0). After subculturing the callogenic explant on MS medium containing TDZ (2.0 mg/l) + NAA (0.5 mg/l), 85 % shooting was achieved. Further subculturing on MS medium containing TDZ (2.5 mg/l) + NAA (1.0 mg/l) resulted into shoots with maximum number (12) and optimum length (5.4 cm) per explant. Rooting of elongated shoots was achieved on MS medium containing NAA (1.0 mg/l).

Adventitous roots were induced from leaf explant of B. oleracea var. acephala on MS medium supplemented with auxins; NAA, IAA and IBA with concentration of 0.1, 0.5, 1, 1.5 mg/l for each plant growth regulator. Best adventitious rooting was achieved at MS medium containing 1 mg/l NAA with maximum 87 % rooting, 35 roots per explant, 2.986 g Fresh Weight (FW) and0.238 Dry Weight (DW). Effect of sucrose concentration and pH was also studied for adventious rooting at MS medium containing 1 mg/l NAA. MS medium with 3 % sugar strength was recorded as the best medium on which maximum 93 % adventious rooting with 2.23 g FW and 0.1 g DW were recorded. 5.8 were recorded as the optimum pH for induction of adventitious rooting on which 85.5 % rooting; 2.33 g FW and 0.186 g DW were recorded. These roots were sub-cultured in shake flask and increase in biomass was determined after each week, tell maximum biomass per 30 ml media was achieved.

The antioxidant activity of regenerated tissues was determined and optimum scavenging activity was found in seed derived plants (36.58 %) followed by regenerated (27.5 %) and lowest activity (7.8 %) was in AR directly excised from leaf after induction followed callus (23.4 %).

Key Words: *Brassica oleracea var. acephala; organogenesis, Adventitious root culture, Thidiazuron, 6-Benzyladenine, Naphthalene Acetic Acid, Fresh Weight, Dry Weight, Antioxidant activity.*

1. INTRODUCTION

The genus *Brassica* belongs to Brassicaceae or mustard family (syn. Cruciferae), comprise of 350 genera and 3000 species (Cogbill *et al.,* 2010; Musgrave 2000). In Pakistan it is distributed by 92 genera and 250 species (Jafri, 1973) and distinguished by its two-segmented fruit and unique conduplicate arrangement of the cotyledons or first leaves in the seed (Gómez-Campo, 1999; Warwick and Sauder, 2005). This genus comprises of economically important edible and industrial oilseed, vegetable, condiment, and fodder crops (Cogbill *et al.,* 2010; Musgrave, 2000). Most of its members have potential for production of bio fuel, pharmaceutical products and can be manipulated for molecular farming (Gugel and Falk, 2006; Warwick *et al.,* 2007). More than these, the considerable importance of *Brassica* vegetables, *Brassica oleracea*, a highly diversified group of crops grown worldwide (Monteiro and Lunn, 1998) and subdivided into six groups/morph types (Snogerup, 1980) which are, 1) Kale (var. acephala) includes green kale, marrow stem kale, collard; 2) Cabbage (var. capitata) includes headed cabbage, Brussels sprouts, Savoy cabbage and others; 3) Kohlrabi (var. gongylodes); 4) Inflorescence kale (var. botrytis, var. italica) that includes cauliflower, broccoli, sprouting cabbage; 5) Branching bush kale (var.. fruticosa); and 6) Chinese kale (*B. alboglabra*) (Demir & Balkaya, 2005). These different crops are tough to distinguish, except for certain strains of cabbage, broccoli and tropical type cauliflower.

B. oleracea var. *acephala,* a diploid plant with 9 pairs of chromosomes, making total nucleic acid content of about 696-765Mbp (Johnston *et al.,* 2005) is one of the oldest form of cabbage, originated before 2000BC in eastern Mediterranean region as a food crop, from where it spread to other part of the world through travellers and immigrants (Nieuwhof, 1969). It is commonly accepted that the origins of cabbage are North European countries, the Baltic Sea coast, and the Mediterranean region (Monteiro & Lunn, 1998). It is an annual cold season (15-20C^0) crop, grows in soil of neutral pH (6.6-7.5), up to height of 0.4-2 meters, with alternate wavy margined large leaves and terminates with inflorescence of yellow colour. The flower has four sepals, a two celled superior ovary of a single large stigma and style, sex stamens two of which have short filaments (Dikson and Wallance, 1986).

The members of Brassicaceae are highly nutritious; being sources of minerals (Na, Ca, Mg, K, and P were analysed in lamina and petioles by Almeida and Rosa in 1994),

vitamins (Vitamin C, 107mg/100g according to Sikora *et al.,* 2008) and also contains essential amino acids (Rosa, 1999) but have low caloric value (24-34 Kcal/100g) because of low protein content, an average content of fibre (2.5/100 g) (Heimler *et al.,* 2006). All green leafy vegetables contain important dietary cartenoids, but the *B. oleracea* var. *acephala* contain the highest amount of Lutien and ß-carotene (Kopsell *et al.,* 2004). Carotene is yellow, orange and red pigment a lipid soluble plant derived secondary metabolite that along with Chlorophyll a and b harness sunlight and found in plants bounded with specific proteins, while Lutein is an oxygenated xanthophyll (Tracewell *et al.,* 2001). Medicinally the intake of ß-carotene, α-carotene and lutein are useful in prophylactic treatment of pulmonary cancer (Le Marchand *et al.,* 1993) as well as for reducing chronic eye diseases, cataract and age related macular degeneration (Johnson *et al.,* 2000). Other than these, species of *B. oleracea* contain phenolics (flavonoids and hydroxycinnamic acid derivatives) and glucosinolates (Cartea *et al.,* 2008; Vallejo *et al.,* 2004) which are bioactive compounds and have been identified in foliar parts of *B. oleracea* var. *costata* (Ferrers *et al.,* 2007 & 2005) and seeds (Ferreres *et al.,* 2009). The anticancer activity of *B. oleracea* is mainly associated with the presence of two isothiocyanates namely sulforaphane (1-isothiocyanato-4-methylsulphinylbutane) and iberin (1 isothiocyanato-3-methylsulphinylpropane) derived respectively from the precursor Glucosinolates, glucoraphanin and glucoiberin mostly abundant in broccoli, cabbage, kale, cauliflower, watercress and Brussels sprouts (Wang *et al.,* 2005). Furthermore, seeds of Brassicaceae members have been characterized for sinapoylcholine (or sinapine), which is thought to serve as a storage form of choline and sinapic acid for germinating seedlings (Hemm *et al.,* 2003).

Biotechnological tools are important for multiplication and genetic enhancement of the medicinal plants by adopting techniques such as *in-vitro* regeneration and genetic transformations. The biotechnological tools are important to select, multiply and conserve the critical genotypes of medicinal plants (Tripathi and Tripathi, 2003). *In vitro* regeneration that offers a great opportunity for a rapid production of desirable and genetically identical plants (Lazzeri and Dunwell, 1986; Msikita and Skirvin, 1989) as well as an efficient *in vitro* regeneration system is a key step in genetic engineering of the crop (Cao and Earle, 2003) and Plant cell, tissue and organ culture is often an effective system to study the biological significance of bioactive metabolites under *in vitro* conditions, as well as for producing natural products for bioprocess applications

(Paek *et al.,* 2005; Saifullah *et al.,* 2008). The regeneration potential of plants is mitigated by the use of phytohormones, such as kinetin or benzyladenine would enhance shoot proliferation and root formation (Arnison *et al.,* 1990) and various concentrations of auxins such as α-naphthalene acetic acid (NAA), indolebutyric acid (IBA) and indoleacetic acid (IAA) have been evaluated for rooting of *in vitro* regenerated shoots of broccoli and cauliflower (Vandemoortele *et al.,* 1999; Widiyanto and Erytrina, 2001). While Thidiazuron (TDZ), a substituted phenyl urea (N-phenyl N' 1, 2, 3- thiadiazol–5-ylurea) is primarily used as a cotton defoliant (Eapen *et al.,* 1998), and aids in rapid plant regeneration of a number of plant species (Chand *et al.,* 1999; Seelye *et al.,* 1994; Kanakis and Demetriou, 1993; Malik and Saxena, 1992).

Roots of various plants are biosynthetic factories or depots of nutritionally or pharmaceutically potent metabolites such as alkaloids, polyacetylene, sesquiterpenes and napthoquinones (Carvalho and Curtis, 1998). But biotechnology made possible adventitious root culture, postembryonic root that arises from stem, leaves, callus and other non pericycle tissues of old roots, which have the potential for commercial scale vegetative propagation of plants (Li *et al.,* 2009; Esau, 1977; Barlow, 1986) as well as strain improvement and species conservation (Holobiuc and Blindu, 2006) in the world. For example *Hypericum perforatum*, a medicinal plant has been found difficult to automate large scale regeneration of plants from leaves, hypocotyls, stem cuttings and stamens (Zobayed and Saxena, 2003). Goel *et al.,* (2009) obtained shoot organogenesis from *in vitro* grown roots in liquid medium of this specie for consistent and large scale micropropagation.

Reactive oxygen species (ROS) which are produced during normal course of cell metabolism inactivated or neutralized by natural antioxidative defence mechanism. Natural antioxidant mechanisms include antioxidant enzymes i.e. superoxide dismutase, catalase and glutathione peroxidise while some non-enzymatic components i.e. antioxidant vitamins and some antioxidative micronutrients which are taken from the diet. Anyhow, when these natural antioxidative mechanisms are compromised, it leads to oxidative stress (Kanehira *et al.,* 2003).

Oxidative stress has been involved in numerous diseases and disorders (Halliwell, 1995; Rackova *et al.,* 2007) such as cancer (Kinnula and Crapo, 2004), cardiovascular disease (Singh and Jialal, 2006), neural disorders (Sas *et al.,* 2007), Alzheimer's disease (Smith *et al.,* 2000) mild cognitive impairment (Guidi *et al.,* 2006), Parkinsons disease (Bolton

et al., 2000), alcohol induced liver disease (Arteel, 2003), ulcerative colitis (Ramakrishna *et al.,*1997), ageing (Hyun *et al.,* 2006), atherosclerosis (Upston *et al.,* 2003).

Plants that are rich in flavonoids, lignans and related phenolic compounds are ideal sources of natural antioxidants (Kanehira *et al.,* 2003). The antioxidant activity of plant extract is primarily due to phenolic compounds (Yesil-Celiktas *et al.,* 2007). Phenolic compounds are commonly found in both edible and non-edible plants. They are important in the plant for normal growth development and defence against infection and injury. The presence of phenolic compounds in injured plants may have an important effect on the oxidative stability and microbial safety. Although phenolic compounds do not have any known nutritional function, they may be important to human health because of their antioxidant potency (Hertog *et al.,* 1995; Shadidi and Nazck, 1995; Hollman *et al.,* 1996). Biotechnological methods based on *in vitro* culture of tissues and plants are considered to give the possibility of producing standardized material, independent from environmental factors (Grzegorczyk *et al.,* 2007).

DPPH method is considered as a simple, rapid and convenient method, independent of sample polarity for screening of many samples for radical scavenging activity (Koleva *et al.,* 2001). This method is based on the reduction of DPPH in methanol solution in the presence of a hydrogen donating antioxidant due to the formation of the non-radical form DPPH-H. DPPH is a stable free radical and is widely used to assess the radical scavenging activity of antioxidant compounds (Blois, 1958).

Aims and objectives:

1. To find out Thidiazuron (TDZ) induced regeneration potential in leaf explants.
2. To study the effect of different Auxins on adventitious root culture.
3. To elucidate the antioxidant potential of regenerated tissues, adventitious roots and seeds.

Figure 1: *Brassica oleracea var. acephala*

2. Literature Review

2.1 *Brassica oleracea* var. *acephala*

Brassica oleracea L. is a significant Cole (derived from Latin word *caulis*, means *stem or cabbage*) crop due to its use as vegetable and has totally cross-fertile cultivars which makes a group of broadly different types on the bases of leaf colour, size and shape (kale, collards, cauliflower, kohlrabi, Brussels sprouts, cabbage and broccoli (Sarikamis *et al.*, 2009; Monteiro and Lunn, 1998). These variations in cultivars are induced by genetic variability as well as by environmental factors (Via *et al.*, 1995). It belongs to the *Brassicaceae* (also called *Cruciferae* because of its sepals arrangement in flower) family comprise of 350 genera, like *Cameline, Sinapis, Brassica, Crambe and Thlaspi* and up to 3,500 species. But of these *B. oleracea* L., *B. napus* L. and *B. rapa* L. are of universal economic importance.

Brassica oleracea var. *acephala* locally, in Kashmir it is called as Karam saag (www.efloraofindia.com) is an important foliage herbaceous plant that is used as vegetable and taxonomically is an oldest form of cabbage but does not form head and nearer to undomesticated cabbage than those cultivated Cole crops. It is cultivated once in a year as cool season, spring or fall crop and matures in about 50-60 days.

The classification of *Brassica* vegetables (*Brassica oleracea* L. spp.) is very difficult. Several species in this genus form a closely weaved net and delimitation of them is of debate (Spooner *et al.*, 2003). To delimitate these Harberd (1972) made a term of ``*cytodemes*`` for a group of plants having constant number of chromosomes and capable to cross-pollinate while those of different *cytodemes* unable to do so. He identified 11 cytodeme of *Brassica oleracea* (2n=18) and made them as subspecies, while 10 by Hanelt (2001) and for classification he followed Helm (1963). In 1935 U, a botanist putt forward a theory in which he showed the evolutionary relationship between cultivated *Brassica* species and presented in a triangular diagram called U triangle. He stated that the genomes of three ancestral diploid (*Brassica nigra*, *Brassica oleracea* and *Brassica rapa*) species of *Brassica* combines together and form three new, amphidiploid frequently used vegetables and oil yielding species (*Brassica carinataI*, *Brassica juncea* and *Brassica napus*) (Jules, 2009; Warwick and Black, 1993). Molecular work confirmed these findings and established the male and female contributions of each primary species, using chloroplast DNA restriction site data (Palmer *et al.*, 1983). AFLP

markers analyses by Christenses *et al.,* (2011) revealed the diversity among 17 accessions of European kale.

It has been proved that today's head cabbage have been descended from wild *Brassica* that does not form head (Dikson and Wallance, 1986). The accepted origin of *Brassica* is countries of north Europe and coast of Baltic Sea (Monteiro and Lunn, 1998). While Vural *et al.,* (2000) consider the Mediterranean region as origin of cabbage, similarly Zhokovsky consider the Van region in Anatolia as origin of white head cabbage (Balkaya *et al.,* 2005) and it is believed that use of Kale crop as food started before 2000BC. In 350 BC Theophrastus described a form of kale called savoyed. Centuries after these days this vegetable was introduced in many parts of the world by immigrants and travellers (Nieuwhof, 1969).

2.2 Uses

The use of plants belonging to *Brassica* genus varies according to the type of specie and has remained in use since Greeks and Romans time as they (Cato, Theophrastus, Columella, Pliny, Dioscorides and many other) have described these plants for culinary and medicinal purpose. It is believed that during construction of China Great Wall the labourers used sauerkraut (a dish made of Chinese cabbage) to overcome devastating effect of scurvy because of eating rice only (Turgeon, 1977). *Brassica* crops are consumed by humans throughout the world and considered a nutritious food of humans, mostly consumed by poor people of Pakistan, India, and China, etc. On the bases of its use these plants have been categorised as forage and vegetable (*B. oleracea* and *B. rapa*), oilseed (*B. napus*) and condiment (*B. carinata*, *B. nigra* and *B. juncea*) crops due to use of their different parts like buds, leaves, seeds, stems, roots and inflorescence. It is generally considered that Cole crops meal is more liked by women than men (Oxford Encyclopedia of Food and Drink in America, 2004; Carvalho, 2010). Before the introduction of potato to Europe (when it was native Western Asia) turnip (*B. rapa*) was used as staple food, livestock feed and in folk medicine its powdered seeds and ointment of flower were used to cure Breast tumour, cancer and skin cancer (Nieuwhof, 1969).

In 19th century *Brassica* crops were suggested for medicinal uses and these crops were used for treatment of ailments like Diarrhoea, Headache, celiac and stomach trouble, deafness and gout. The juice of fresh cabbage was considered as hollowed traditional remedy and was used as anti-asthmatic and antidote of toxic mushrooms, also for rinsing

the mouth against hoarseness, treat warts and leaves of cabbage used to make blister to treat inflammation (Patel *et al.*, 2011). Similarly red cabbage fresh juice was also used in bronchitis, chronic cough and asthma. In traditional medicine antibacterial activity of turnip roots and used against common cold (Duke, 1983). According to Sousa *et al.*, (2008) plants belonging to *Brassica* genus are related to cure carcinomas, particularly of colon, rectum and stomach, also prevents from cardiovascular diseases (Sousa *et al.*, 2008; Traka and Mithen, 2009).

Brassica`s versatile use and its ability to adopt its self to any growing system, proves its importance for centuries. Though due introduction of highly diversified vegetable salad throughout the year in the developed countries have made it less attractive to consumers. But due to its nutraceutical potential it still has a market value in developed countries while in less developed countries it is still of more importance due to low price value and abundance for consumers (Monteiro and Lunn, 1998).

2.3 Phytochemistry

Plants and animal provides food to survive in the planet, but in parallel to food they also contains biologically active chemicals called phytochemicals, like Phenols, Flavonoids, carotenoids, sulfer containing organic compounds, vitamins, etc and chemistry of these phytochemicals called Phytochemistry (Plumb *et al.*, 1997). The use of phytochemicals for medicinal purposes is gradually increasing in the world (Gieslene *et al.*, 2000). The intake of these phytochemicals in food reduce risk of cancers, cardiovascular and other fatal diseases (Art and Hollman, 2005; Kaur and Kapoor, 2001; Scalbert *et al.*, 2005; Vita, 2005).

Brassica vegetables contain phytochemicals (folic acids, selenium, phenolics, glucosinolates, vitamin C and carotenoids) which have the ability to promote health and protect from disease risk (Femina *et al.*, 1998; Kushad *et al.*, 1999; Conaway *et al.*, 2001; Fimognari *et al.*, 2002).

Glucosinolates, β-thioglucoside N-hydroxysulfates are sulphur containing metabolites also called S-glucopyranosyl thiohydroximates or (Z)-(or cis)-N-hydroximinosulfates esters and structurally consist of β-D glucose moiety that is covalently bonded to sulphated thiohydoximate and side chain that varies according to 130 different glucosinolates types (Fabre *et al.*, 2007; Moreno *et al.*, 2006). They are anionic, hydrophilic compounds that plays role in curing cancer and other degenerative and

chronic diseases (Fahe *et al.,* 2003). Crucifers are important source of glucosinolates (Ugolini *et al.,* 2008). Sinigrin was reported by Ugolini *et al.,* (2008) in black, Indian and Ethiopian mustard and predominant glucosinolate, glucosinalbin was reported in yellow mustard. Kajaer (1980) reported that sinalbin occurs in trace amount in *B. napus* seeds while Bergmann (1970) and Josefsson (1970) reports higher amount of indolyl glucosinolate in vegetative parts of *Brassica* vegetables while lacking in seed. The quality and quantity of glucosinolates is specie also dependants on varies plant parts, environmental conditions, age of plant and agronomical factors (Tiedink *et al.,* 1988; Kushad *et al.,* 1999; Vallejo *et al.,* 2002 & 2003b; Borkowski *et al.,* 2008).

Another class of secondary metabolites that exist in *Brassica* plants is Phenols or Phenolics, a class of aromatic organic compounds that has one or more hydroxyl groups attached to an aromatic hydrocarbon or benzene ring (Scott, 2007), Phenolics varies from single cyclic aromatic compound of low molecular weight and simple to complex and large tannins and derived poly phenols, and produced in plants through the shikimic acid pathway in which PAL (Phenylalanine ammonialyase) plays a key role (Crozier *et al.,* 2006; Pereira *et al.,* 2009). Due to its ability to protect humans from heart diseases and cancer because of their antioxidant activity, phenolics have gained significant attention. The *Brassica* vegetables have varying phenolic composition and been recently found in the said genus and till to date the phenolics profile of different *Brassica* vegetables have been established (Cartea *et al.,* 2011).

The occurrence of phenolics in Cole cops have been reported in many studies (Velasco *et al.,* 2010; Ferreres *et al.,* 2005; Vallejo *et al.,* 2004; Llorach *et al.,* 2003; Nielsen *et al.,* 1993). While the amount of phenolic compounds varies according to environment as well as the method used to analyse the content of phenols, so the comparison for content of phenols among *B. oleracea* crops is difficult (Cartea *et al.,* 2011). Studies have shown that cumulative content of phenols of tronchuda cabbage (Ferreres *et al.,* 2006) is different from that of savoy cabbage (Martinez *et al.,* 2010). Studies of Ferreres *et al.,* (2006 & 2005) and Sousa *et al.,* (2005 & 2007) have shown the kaempferol, quercetin and phenolic acid derivatives from seeds, sprouts, internal and external leaves of tronchuda cabbage. Recent reports used another approach, antioxidant activity analysis (PAL activity) for claiming the presence or absence of phenols in plants and claimed the direct proportion of phenols to antioxidant activities (Moreno *et al.,* 2006; Llorach *et al.,* 2003; Vallejo *et al.,* 2003a). Heimler *et al.,* (2006) evaluated the phenolics in many *B.*

oleracea crops. He found broccoli and kale with highest content of phenolics as well as flavonoids.

Instead of these two major phytochemicals *Brassica* crops also contain flavonoids and brassinosteroids. Flavonoid is a class of phytochemicals which are polphenolic in nature and consist of two aromatic rings of 15 carbons joint covalently by three carbons (C6-C3-C6). Isorhamnetin, Quercetin and kaempferol are the major flavonols found in conjugation to glucose in *Brassica* crops (Mean and Mohamed, 2001). Flavonoids have been found in several edible *Brassica* crops like pak choi (*B. campestris*), cauliflower, tronchuda cabbage and broccolli (*B. oleracea*) as well in turnip tops (*B. rapa*) (Llorach *et al.,* 2003; Vallejo *et al.,* 2004; Ferreres *et al.,* 2005&2008; Rochfort *et al.,* 2006; Romani *et al.,* 2006; Harbaum *et al.,* 2007).

Kurilich *et al.,* (1999) studied edible portion of 50 broccoli and 13 cabbage, Brussels sprouts, cauliflower and kale accession and reported ß-carotene, *α*--carotene, γ-tocopherol, and ascorbate using reverse phase and sample HPLC analysis systems. Out of these the highest concentration of vitamins was observed for kale, so he concluded that the concentration of phytochemicals in vegetables is genotype dependant. Both ß-carotene and *α*—carotene are precursors of vitamin A.

Brassinosteroids composed of a common 5-α-choletan skeleton, that found in *Brassica* crops are poly-hydroxy steroids and up to 42 different types (on the basis of type and arrangement of functional groups around the skeleton) have been characterized (Fujioka and Sakurai, 1977; Yokota, 1997). Grove *et al.,* (1979) reported brassinolide, a steroidal lactone in pollens of *B. napus*. Medicinally brassinosteroids are effective against HSV1 (herpes simplex virus 1), measles virus and RNA viruses as well as it also inhibits the proliferation of prostate and breast cancerous cell line in a micro molar amounts (Wachsman *et al.,* 2000 & 2002).

2.4. Tissue culture

Generally Plant tissue culture technology is playing increasingly important role in field of biotechnology. It is defined as culture of different plant tissues, organs 0r somatic cells under controlled laboratory conditions to identical plantlets called tissue culture (Dobranszki and Da Silva, 2010). It is a technique not a technology. The plants are traditionally raised from seeds or cuttings. Seeds are commonly infected by fungal, bacterial, viral and mycoplasmal pathogens (Philip *et al.,* 1992). A special characteristic

of plant cells and meristems in which they retain a latent capacity to produce a whole plant is called totipotency (Reinert and Backs, 1968; Vasil and Vasil, 1972; Verdeil *et al.,* 2007; George, 2008). The technique of tissue and organ culture is used for rapid multiplication of plants, genetic improvement of crops, obtaining disease free clones and preserving valuable germplasm (Bhojwani and Razdan, 1992). Micropropagation is also used to promote germplasm storage for maintenance of disease-free stock in controlled environmental conditions (Withers, 1980) for longer term via cryopreservation technique (Kartha *et al.,* 1980). Conventional breeding has added some elite cultivars while *in vitro* technology can serve as an alternate means for further genetic upgrading and its successful application depends largely on a reliable plant regeneration system (Mandal *et al.,* 1995).

Plant tissue culture have opened new field that is called as Bioprocess technology that deals with (1) increased production of secondary metabolites like, alkaloids, pharmaceutics, nematocidal compounds, and also some novel compounds, (2) scale up of cultures in bioreactors, and (3) transgenic plants, cells or tissues culture in bioreactor to produce vaccines etc. These developments have far-reaching implications in the improvement of medicinal plants as well. Similarly through this technology scientist have introduced new plants naturally native to Europe or other areas, like strawberry (Smith and Drew, 1990) and Pineapple (Drew, 1988).

In vitro techniques offer the possibility of rapid clonal multiplication of elite plant species, allowing production of genetically stable and identical progeny (Hu and Wang, 1983). *In vitro* propagation is an alternative method to traditional propagation (Abbasi *et al.,* 2007; George and Sherrington, 1984). *In vitro* culture offers improvements over traditional vegetative propagation because of faster rate of multiplication.

Plant regeneration has been increasingly optimized via organogenesis and somatic embryogenesis using various explants; with tissue culture improvements focusing on factors such as age of the explant, genotype, and media additives. The production of haploids and doubled haploids using microspores has accelerated the production of homozygous lines in the *Brassica* species. Somatic cell fusion has facilitated the development of inter-specific and inter-generic hybrids in the sexually incompatible species of Brassica. Crop improvement using somaclonal variation has also been achieved. Table 1 shows the previous reported work done on various cultivars of *B. oleracea* L.

Table 1**:** *In vitro* regeneration of different cultivars of *B. oleracea* L.

Cultivar	**Explant**	**Medium used**	**Phytohormones mg/L**	**References**
B. oleracea subsp. *Italic* cv. Green marvel	Cotyledonary	MS	BAP+NAA(3+1 mg/L)	Ravanfer *et al.*, 2011
Broccoli, savoy cabbage, red cabbage and caulifllower	Hypocotyle, Root & Cotelydon	MS	KIN+IBA (1+0, 0.1 & 0.2mg/L)	Pavlovic *et al.*, 2010
Kale	Cotyledon& hypocotyl	MS	BAP+NAA (3+1 mg/L)	Dai *et al.*, 2009
B. oleracea var. capitata	Hypocotyle & cotyledon	MS	BAP	Munshi *et al.*, 2007
B. oleracea var. *italic*	Leaf explant	MS	BAP + KIN	Cao and Earle, 2003
B. oleracea var *acephala*	Leaf explant	MS	Zeatin+NAA (5+1, 0.1, 0.05)	Hosoki *et al.*, 2003
B. oleracea var *acephala*	Leaf explant	MS	BAP+KIN ()	Donato *et al.*, 2002
B. oleracea var. *botyris*	Vegetative and floral	MS	BAP + GA3(3+0.01 me/L)	Bhalla and Weard, 1999
White cabbage	Meristem tips	MS	KIN (2.56+12.8 mg/L)	Walker *et al.*, 1980
B. oleracea var. *acephala*	Auxillary &flower buds, pedicals	MS	BAP+NAA (4+0.1mg/L)	Peng-fang *et al.*, 2003
B. oleracea var *italic*	Hypocotyl	MS	BAP	Kim and Botella, 2002

2.5 Adventitious root culture

The post- embry onic roots that arise from unusual sites like callus, leaves, stem old roots and are different in form from lateral roots called adventitious roots (Smart et *al.,* 2003; Zobel, 1986). And its formation is a complex and distinctive process, which is vital step in plant propagation process (Ford *et al.,* 2002). Barlow (1986) defined roots arising from part of plant that does not originates from embryonic root, means that arises from part of shoot. Anatomical studies have shown that these roots originates from cells around cambial zone or parenchyma of phloem e.g. cuttings of Malus (De Klerk, 1995).

The induction of these roots is of key importance in successful propagation of regenerated and vegetative (cuttings) propagation. It`s induction is affected by different factors like genotype, PGRs (endogenous and exogenous), media used and also by environmental factors (Calamar and De Klerk 2002). Several studies have been taken to investigate origin of adventitious roots in plants (Falasca *et al.,* 2004; Belehu et *al.,* 2004; Smart et *al.,* 2003; Mahlstede and Watson 1952; King and Stimart 1998). Caldero´n Baltierra *et al.* (2004) used globules shoots in Eucalyptus and found roots formation primarily at medulla. Soh *et al*. (1998) suggested that adventitious root originate from dedifferentiated parenchymal cells, around the vascular bundles. Falasca and Altamura (2003) studied three ecotypes of Arabidopsis thaliana and found pericycle tissues in hypocotyls as origin of adventitious roots.

Kevers *et al.* (1997) studied adventitious rooting in many woody plants, but mainly in Malus and recognised three different i.e. induction, initiation and expression phases in adventitious rooting. In induction phase no clear morphological changes can be seen while just biochemical and molecular events take place. In second phase Cell division occurs and meristem of roots develops which form root primordia. In third phase an outgrowth emerges from cutting occurs.

Adventitious roots are of key importance plant propagation industry because successful propagation of plants depends on root induction (Ford *et al.,* 2001). On the other hand it was successfully applied on medicinally valuable plants like Taxus baccata, which contain taxanes or diterpene amids which have a potential to cure cancer.

Akashia *et al.* (2005) successfully established adventitious root culture of Iris germanica and obtained higher amount of isoflavones from these roots.

Adventitious root culture is the unique technique which renders the secondary metabolites in huge amount and it fulfils the global demand in field of medicine, agriculture, drug production, pigment production, dye production and so on. Root cultures can be used to study effect of gravitropism, growth regulators, minerals and vitamins. More over these in vitro root cultures can be used to study carbohydrate metabolism and differentiation of root tips. The main advantage of in vitro root cultures is rapid growth rate, comparatively to cell culture its handling and maintenance is easy, show a low level of clonal variations, so large number of identical experimental tissues can be produced (Nagarajan *et al.*, 2011).

The establishment of adventitious root culture is multifaceted process that is influenced by numerous factors, including both endogenous factors that include phytohormones, and environmental factors, such as light and wounding. While it's molecular mechanism is still unclear (Sorin *et al.*, 2005).

A key role is played by PGRs (Blakesley, 1994) that may also interact with other internal factors or with external (environmental) stimuli i.e. light. Fett-Neto *et al.* (2001) used *Eucalyptus saligna* and *E. globules* and reported that auxin act in mutual association with light i n the development of adventitious roots. Recently, Niemi *et al.* (2005) observed that mycorrhyza and adventitious root formation in Scots pine (*Pinus sylvestris*) effected by light sources and different spectra.

Arabidopsis thaliana has been shown to be an important model plant for understand the molecular events that occurs in control of environmental signals to mediatd adventitious root initiation.

According to King and Stimart (1998), in response to auxin, several ecotypes of *A. thaliana* have different capacities to induce adventitious rooting from hypocotyl explant and he concluded that several independent genes may control low and high rooting responses in an additive manner. Konishi and Sugiyama (2003) studied temperature-sensitive mutant model plants of *Arabidopsis* with altered adventitious rooting ability.

Carbohydrates are basic metabolic units and provide energy for plant cells. The accessibility of which is usually considered entirely as energy need and carbon frame also induce root formation (Luciano *et al.*, 2004).

As sugar plays vital role in repressing the photosynthetic gene s expression (Sheen, 1990) also it interact with ethylene and abscisic acid signals (Leon and Sheen, 2003). So

Borisjuk *et al.* (1998) observed the best rooting response in the presence of sucrose. This is further supported by Cheng *et al.* (1992) work in which he reported that 2- 3 % of sucrose is useful for rooting in Eucalyptus sideroxylon.

Beside from carbon (Sugar) sources, medium pH also plays important role by affecting the developmental stages of the plants. In "acid growth hypothesis" it has been stated that plants cell modifies their expansion by changing cell wall extensibilty according to the pH of surrounding environment (Cosgrove, 1999). Several evidences favour supposition in expanding leaves or in shoot coleoptiles (Rayle and Cleland, 1992; Peters *et al.,* 1998; Van Volkenburgh, 1999; Kotake *et al.,* 2000). This hypothesis of roots has been studied only on limited scale in root induction (Peters and Felle, 1999; Tanimoto *et al.,* 2000; Walter *et al.,* 2000). Thus, the present research work was design to find out the effects of carbon source and pH of medium on adventitious formation from *Brassica oleracea* var. *acephala* leaf explants.

2.6 Anti-oxidant activity

2.6.1 Antioxidants

Compounds of synthetic or natural origin that stop or hold-up the oxidation of substrates (i.e. mostly lipids, but can also be a DNA molecule, carbohydrate or protein) even if the antioxidants are present in a considerably lesser amount comparatively oxidized substrate called anti-oxidants (Halliwell, 1994). Antioxidants are used to maintain quality of food mainly by preventing lipid constituent ionic oxidative deterioration and also protect the human body from toxic effects of free radicals as well retard the progress of many chronic diseases (Gulcin *et al.,* 2004). *Brassica* vegetables contain higher amount of antioxidant which make these more attractive to consumers. There are two types of antioxidants found in *Brassica* vegetables, water soluble (Phenolic compounds, Vitamin C&E and folic acid) and lipid soluble (Carotenoids) antioxidants. The content of antioxidant in *Brassica* vegetables varies with genotypes, plant part, environmental factors, and post-harvest treatments as well as cooking methods (Soengas *et al.,* 2011). Restriction on the use of synthetic antioxidants due to their probable side-effects has increased the demand of natural antioxidants (Velioglu *et al.,* 1998). Antioxidant activity of many phenolic compounds, including flavonoids, has attracted considerable attention and reported to be more powerful antioxidants than vitamins C, E and β-carotene which are largely in routine use. Vegetables and fruits are also reported to decrease the risk of

degenerative diseases and could have a protective effect against oxidative stress (Vinson *et al.,* 1998). Antioxidants, such as vitamin A, vitamin C, vitamin E, carotenoids, polyphenolic compounds and flavonoids are found in plenty in vegetables and fruits and these antioxidants control the free radical damage, reduce the risk of chronic diseases while atherosclerosis can be prevented by the consumption of dietary antioxidants from these sources (Barros *et al.,* 2007).

2.6.2 Antioxidant Activity Determination Methods

Antioxidant properties have been studied in several plant species for the development of natural antioxidant formulations in the areas of food, medicine and cosmetics (Miliauskas *et al.,* 2004). A number of *in vitro* methods have been developed for estimation of antioxidant activity that is grouped to two main types:

1. Hydrogen ion transfer reactions
2. Electron transfer reactions (Huang *et al.,* 2005).

This method`s diversity is due to the complexity of the analyzed substrates, often mixtures of dozens of compounds having different functional groups, polarity, and chemical behavior (Szabo *et al.,* 2007).

3. Materials and methods

All the experimental work was conducted at Plant Cell and Tissue Culture Lab, Department of Biotechnology, Quaid-i-Azam University, Islamabad.

3.1 Germplasm and surface sterilization

Seeds of *B. oleracea* var. *acephala* were obtained from Plant Genetic Resource Institute (PGRI), National Research Centre (NARC) Islamabad. These seeds were surface sterilized according to established protocol of Abbasi *et al.* (2010) i.e. seeds were immersed in 70 % ethyl alcohol for ~3 min, then rinsed with autoclaved distilled water followed by mercuric chloride (0.1%) treatment for ~1 min and finally 3 times wash with autoclaved distilled water.

3.2 Glassware and other equipments used

Flasks, beakers, volumetric cylinders, petri-dishes, scalpels, blade holders and cutters used in the experiments were washed 3 times with distilled water and autoclaved at 121 ^{0}C for 15 min.

Electric weighing balance (Shimadzu, BL-2204) was used for taking appropriate amount of chemicals. To dissolve the medium ingredients hot plate magnetic stirrer (VELP Scientifica) was used and to uniformly distribute the agar in medium boiling was done in microwave oven. To inoculate the flasks aseptically, laminar flow hood (SHC 4A1) was used. Incubator shaker (Innove 43R) was used for establishment of adventitious root culture.

3.3 Source of explants

Seed germination medium or MS0 (PGRs free), a Murashige and Skoog (1962) medium was prepared by taking 4.4g/l MS salts (*Phytotech* laboratories), 30 g/l sucrose and 8 g/l agar (Agar technical LP0013; Oxide, Hampshire England). All these ingredients were mixed, pH was adjusted ~5.8 and media (30 ml) was poured in to each 100 ml flasks and autoclaved at 121 ^{0}C for 20 min. After overnight cooling, autoclaved medium was inoculated with surface sterilized 5 seeds per each flask and transferred to growth room (25±1 ^{0}C). 100% seed germination frequency was observed on third day and 7 days old cotyledon explants was excised for experiments.

3.4 *In vitro* regeneration

3.4.1 Physical Parameters in Lab

All media were autoclaved at 121 ^{0}C for 20 min and pH of all the media was adjusted in the range 5.6±0.2. All cultures were maintained in a growth room at 25 ± 1 ^{0}C under a 16/8-h light/dark photoperiod with light provided by cool-white fluorescent tubes at an intensity ranging from approximately 40 to 50 μmol $m^{-2}s^{-1}$.

3.4.2 Inoculation of leaf Explants

For aseptic conditions, inoculation was carried out inside laminar flow hood. Prior to inoculation it was sprayed with 95% ethanol. The MS medium supplemented with different PGRs (TDZ, NAA and BAP) in different concentration was poured in 100ml flasks and was autoclaved along with, scalpels, blade holder and distilled water. Before starting the experiment all these were treated with UV light (GKL-511; 50Hz, 19w) for 15 min in laminar flow hood. Before inoculation hands were rinsed with soap, dried and then washed with 70 % ethanol.

Leaf explants were excised from 7 day old *in vitro* seed derived plant and cut into reasonable sized pieces for inoculation. These leaf segments were transferred to MS media with different concentrations and combinations of different PGRs and MS0 was used as control. 5 explants were inoculated per 100 ml flask. After 3 weeks of inoculation % responded explants was recorded. The responses (callus formation, shoot/root organogenesis etc.) of explants were recorded on the basis of visual observation.

3.4.3 Sub-Culturing

Callus was sub-cultured repeatedly to maintain its viability and to evaluate its organogenic response. Sub-culturing was performed in Laminar Air flow hood, by cutting the callus into slice shaped thin pieces in sterile petri-plates and then inoculation on fresh MS media. After sub culturing, % shooting was recorded, number of callus responded to shooting divided by total number of callus per flask. The data was taken in triplicate.

3.4.4 Shoot proliferation and shoot elongation

Explants resulting into indirect organogenesis (shoots through callus formation) were subsequently transferred aseptically to shoot proliferation and shoot elongation medium

in Laminar flow hood and maintained in growth room under the same conditions described above and data was recorded after 20 days of sub culturing as number of shoots per explant and mean shoot length.

3.5 Adventitious root culture

3.5.1 Plant Materials

Young and fresh leaf explants were selected from *in vitro* seed derived plantlets for adventitious rooting and were cultured on MS media (Murashige and Skoog, 1962) supplemented with different concentration of auxins (NAA, IAA and IBA).

3.5.2 Culture Medium

MS media of full strength were prepared and supplemented with auxins NAA, IAA and IBA (MERCK Germany) as plant growth regulators (PGRs). These PGRs were added to medium alone. The pH was adjusted to ~5.8 by addition of 0.1 M NaOH or 0.1 M HCL; then 0.8 % agar was added, boiled till the appearance of crystal white colour and poured into 100 ml flasks. After pouring, the medium was autoclaved at 121 ^{0}C and 15 Psi for 15 min.

3.5.3 Effect of different auxins at various concentrations on induction of adventitious roots from leaf explant

Leaf explants were inoculated (5 explants/flask) abaxially on MS medium supplemented with NAA, IAA or IBA (0.1, 0.5, 1.0 and 1.5 mg/l) concentrations. The inoculated medium was kept in growth room for 4 weeks and 25±1 ^{0}C and 16/8-h photoperiod. These treatments were applied in triplicates.

The initiation of root from explants was recorded on the basis of visual observations. Results (percent rooting, fresh and dry biomass of roots in grams) were taken after 4 weeks of inoculation and expressed as mean ± standard deviation. For determination of fresh biomass, adventitious roots were excised from explants, washed to remove agar and then blotted against filter paper and allowed to air dry and weighed. These roots were dried in oven for 24 h at 60 C^0 to get dry biomass.

3.5.4 Effect of sucrose concentration on induction of adventitious roots from leaf explant

After finding the optimum responding auxin and its concentration, further experiments were designed to study the effect of sucrose concentration on adventitious root induction

from leaf explants. The young and fresh leaf explants were inoculated on MS medium supplemented with NAA (0.5 mg/l) having different sucrose concentrations (1, 2, 3, 4, 5, 6, and 7 %). The results (percent rooting, fresh and dry biomass) were recorded as described above.

3.5.5 Maintenance of adventitious root culture

Adventitious roots were excised under aseptic conditions and sub cultured for 4 weeks on MS media supplemented with auxins (NAA, IAA and IBA). After induction, adventitious roots were transferred to 100 ml-Erlenmeyer flasks containing MS liquid media supplemented with various auxins in different concentration. And these flasks were incubated in shaker incubator (Innove 43R) in 24 h dark conditions at 25 ^{0}C and 110 rpm.

Adventitious roots growth kinetics was studied in shake flask culture by taking sample of roots on every 5th day of inoculation to measure increase in biomass, determine pH and conductivity of used medium. Dry biomass was measured by oven-drying hairy roots at 60 ^{0}C for 24 h. The pH and conductivity were measured in the biomass-free culture medium with digital instruments.

3.6 DPPH Free Radical Scavenging Assay

The method of Amarowicz *et al.,* (2004) was used to evaluate the capability of prepared extracts to scavenge free radical DPPH° (α, α-diphenyl-β-picrylhydrazyl). Briefly, 2.0 mg of plant tissue extracts were dissolved in 4 ml of methanol and then added to methanolic solution of DPPHº (1 mM, 0.5 ml). The resulting mixture was vortexed for 15 sec and then left to stand for 30 min at room temperature. The absorbance of the resulting solution was examined spectrophotometrically at 517 nm. A methanolic solution of DPPHº that had decayed and hence no longer exhibited purple color (i.e. 2 mg of butylated hydroxyanisole (BHA) dissolved in 4 ml of methanol with 0.5 ml of DPPHº solution added) was chosen for background correction, instead of pure methanol. Finally the radical scavenging activity was calculated as percentage of DPPHº discoloration using the equation;

$$\% \text{ scavenging DPPH}^{o} \text{ free radical} = 100 \times (1-A_E/A_D)$$

Where A_E is absorbance of the solution, when extract has been added at a particular level and A_D is the absorbance of the DPPHº solution with nothing added.

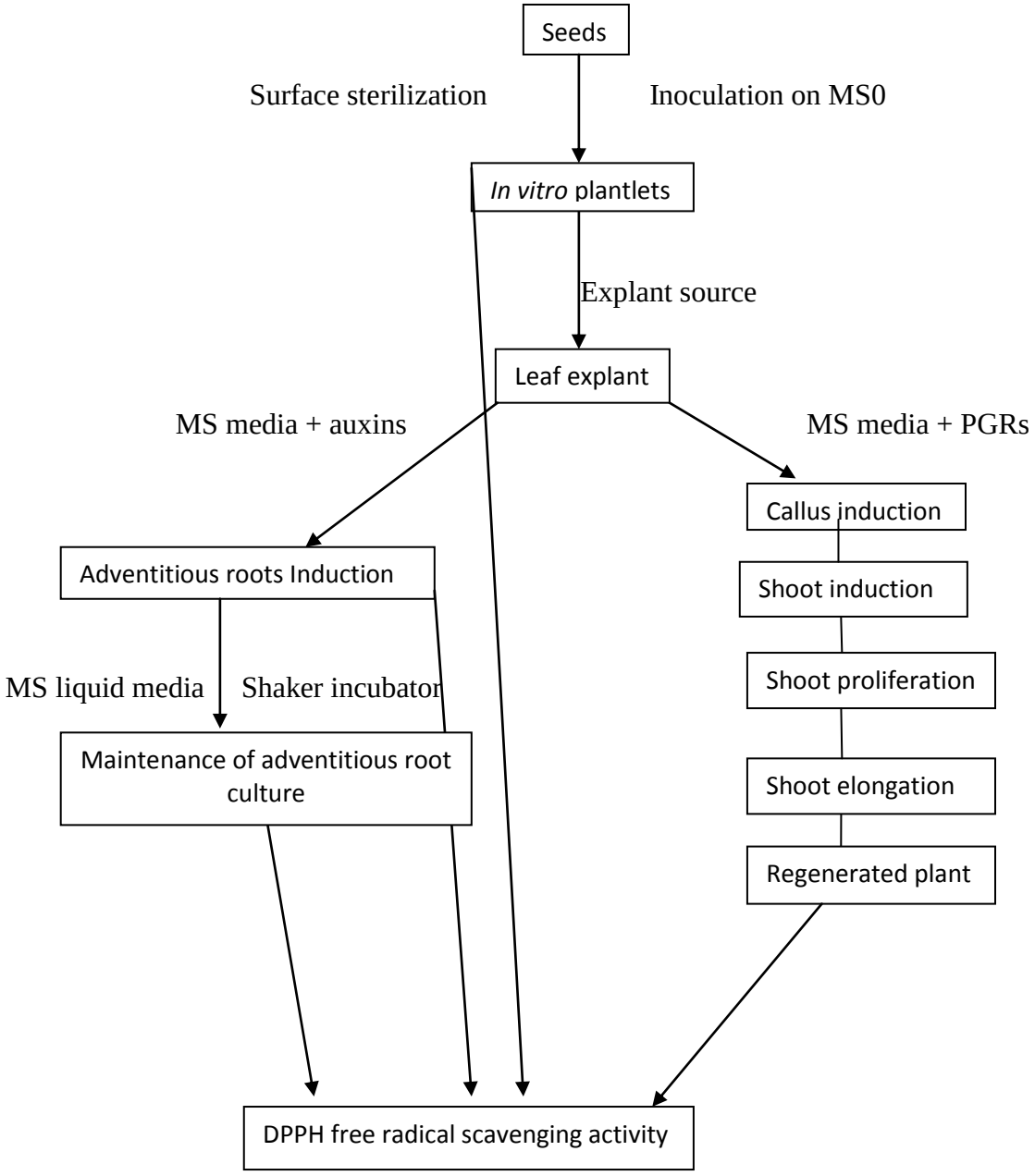

Figure 2: complete experimental work in a flow chart.

4. RESULTS

Objectives of the present study were to evaluate the regeneration potential, to establish adventitious root culture and to determine antioxidant potential of *in vitro* regenerated tissues of *Brassica oleracea* var *acephala*. Incubation of cotyledon explants on MS media supplemented with different concentrations and combinations of PGRs showed callus induction, indirect shooting and direct shooting. On the other hand cotyledon explant incubated on media containing different auxins induced adventitious roots which were further transferred to liquid MS media containing flasks and placed in shaker incubator for 6 months.

4.1 Seed germination and explant source

Seeds of *Brassica oleracea* var. *acephala* were obtained from Plant Genetic Resource Institute (PGRI), Islamabad. These were surface sterilized with 70 % etha nol for 60 sec followed by immersion in 0.1 % (w/v) $HgNO_3$ solution for 2 min and finally rinsed three times in sterile distilled water. PGRs free Murashige and Skoog media (MS0) with 3 % sucrose and 0.8 % agar as solidifying agent was used for seed germination and ~ 95 % germination was observed at day third. Leaf explant was excised from 7 days old seed derived plantlets and cultured on MS media supplemented on various concentrations of PGRs (TDZ , NAA and BAP).

4.2 *In vitro* regeneration

4.2.1 Callus formation

Leaf explants obtained from *in vitro* germinated seeds were cultured on MS medium supplemented with different concentrations of plant growth regulators either alone or in combinations. After four weeks of incubation callus, compact mass of undifferentiated cells was observed by naked eye. The frequency of callus formation from leaf explant on different PGRs was recorded in triplicates and the data was presented in graphical form (Fig. 4a, b)

Fresh compact callus was observed on TDZ alone or in combination with NAA and on BAP in combination with NAA. The highest callus formation frequency 85 % was observed on MS media supplemented with NAA (1.0 mg/l) + BAP (1.5 mg/l) (Fig. 4b) followed by 82 % on TDZ (2.5 mg/l) + NAA (1.0 mg/l) (Fig. 4a). A slow growing granular callus was observed on TDZ 2.5 mg/l with 52 % callus formation frequency. No

callus was observed on MS media supplemented with different concentrations of BAP alone but after four weeks of incubation only enlargement and then browning of the leaf explant was observed while direct multiple shoots were formed in inter-nodal explant. It was noticed that with the increase in TDZ concentration increase in callus formation frequency occurred while a decline was recorded with increase of NAA concentration in combination with TDZ (Fig. 4a). Similarly with incubation of leaf explant on NAA in combination with BAP, an initial increase in callus formation frequency was observed with increase in BAP concentration but gradual reduction in callus formation frequency was observed with increase in concentration of NAA from 1.0 mg/l to 2.5 mg/l in combination with BAP (Fig. 4b). Lowest callus formation frequency, 37 % and 38 % was observed on TDZ 0.5 mg/l alone and NAA 2.5 mg/l in combination with BAP 0.5 mg/l respectively. Slow growing granular callus was observed with a highest frequency of 52 % on TDZ 2.5 mg/l (Fig. 4a).

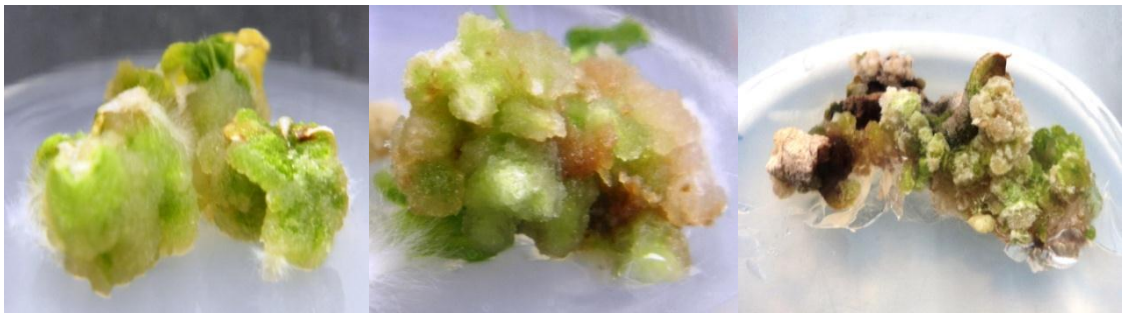

Figure 3: Callus from leaf explant of *Brassica oleracea* var. *acephala*

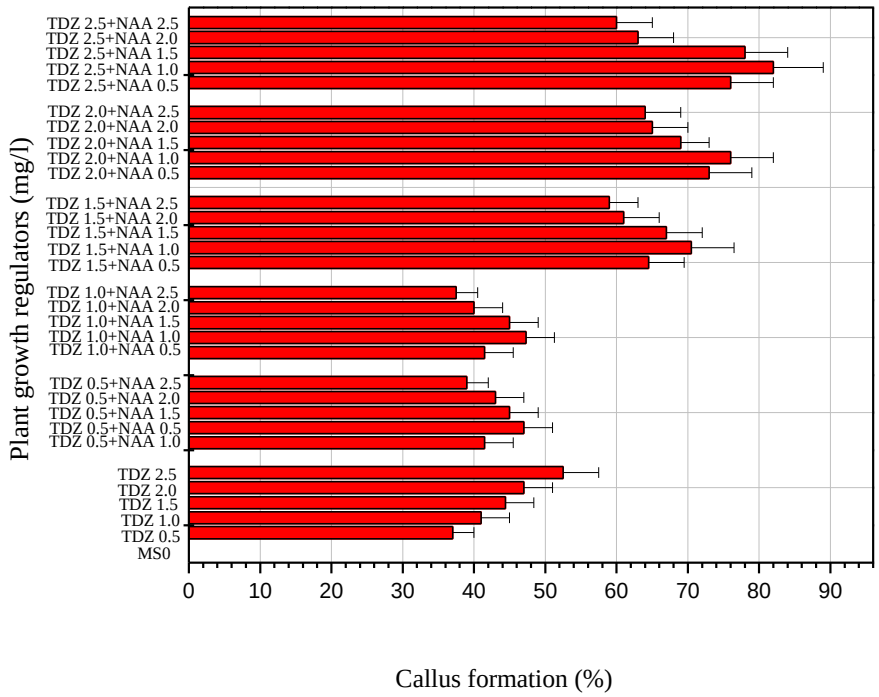

Figure 4a: Callus formation frequency on TDZ alone and in combination with NAA

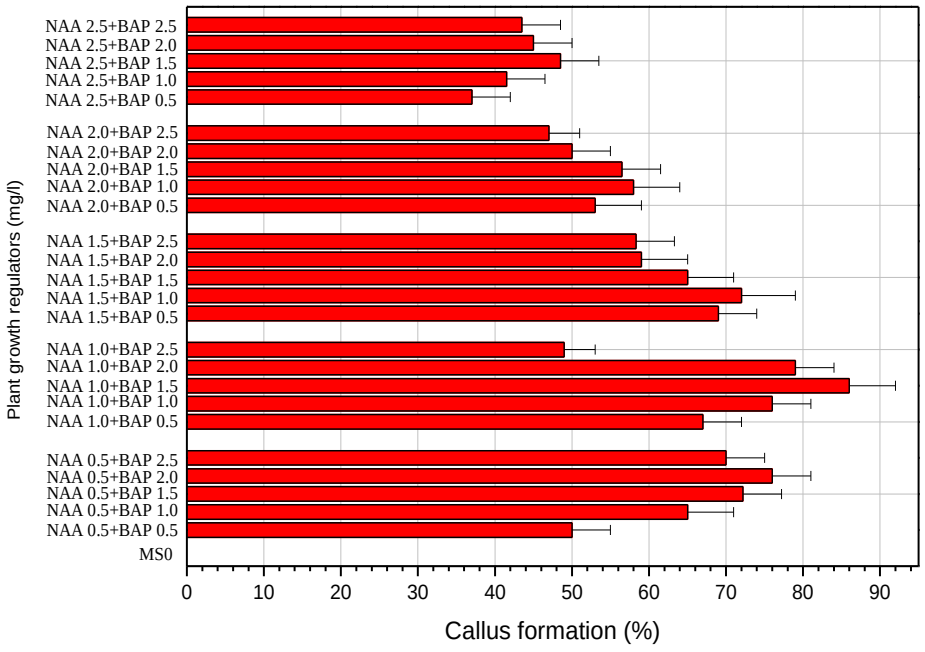

Figure 4b: Callus formation frequency of leaf explant on NAA and BAP in combination

4.2.2 Organogenesis

Following subculturing of 4 week old fresh green callus on MS media having similar composition of PGRs, indirect shooting was observed with highest frequencies of 85 % on TDZ 2.0 mg/l in combination with NAA 0.5 mg/l followed by 83 % on NAA 0.5 mg/l in combination with BAP 2.5 mg/l. The lowest shooting frequency 37 % was observed on TDZ 0.5 mg/l. It was found that with increase in TDZ concentration shooting frequency was increased to 53 % while in combination with NAA a synergistic effect was observed and highest shooting frequency was achieved but with increase of NAA concentration in each treatment with TDZ, reduction in shooting frequency was observed (Fig. 6.a). Fig. 6.b shows shooting frequency in response to NAA in combination with BAP and it shows that the lowest shooting frequency 28 % was observed for NAA 2.5 mg/l + BAP 0.5 mg/l. However, MS media without PGRs failed to regenerate shoots from 4 weeks old callus. So it was concluded that incorporation of auxin (NAA) along with cytokinins enhanced the shoot regeneration frequency.

Figure 5: Indirect organogenesis from leaf explant

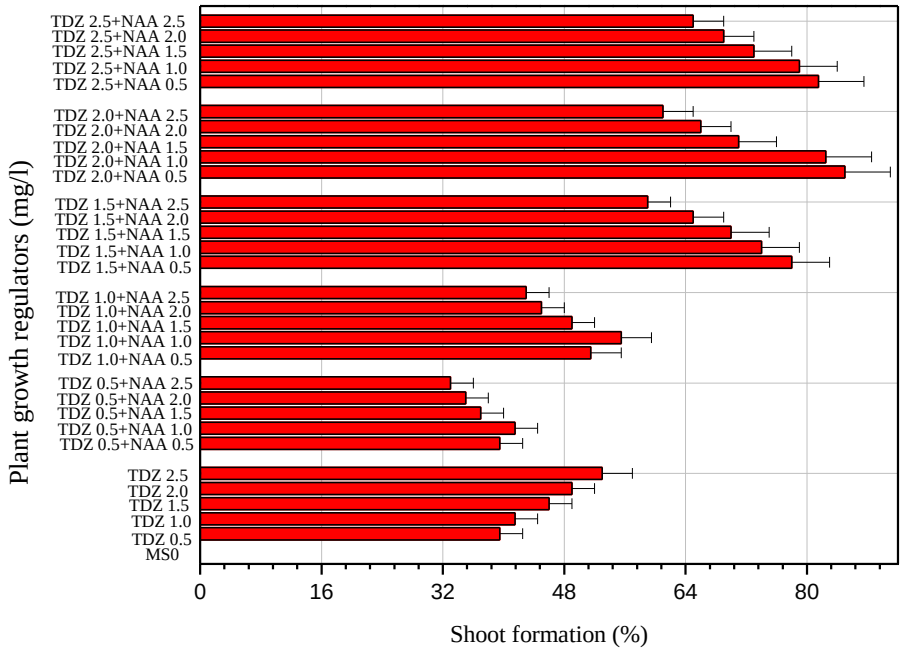

Figure 6a**:** Indirect shooting frequency at TDZ alone and in combination with NAA.

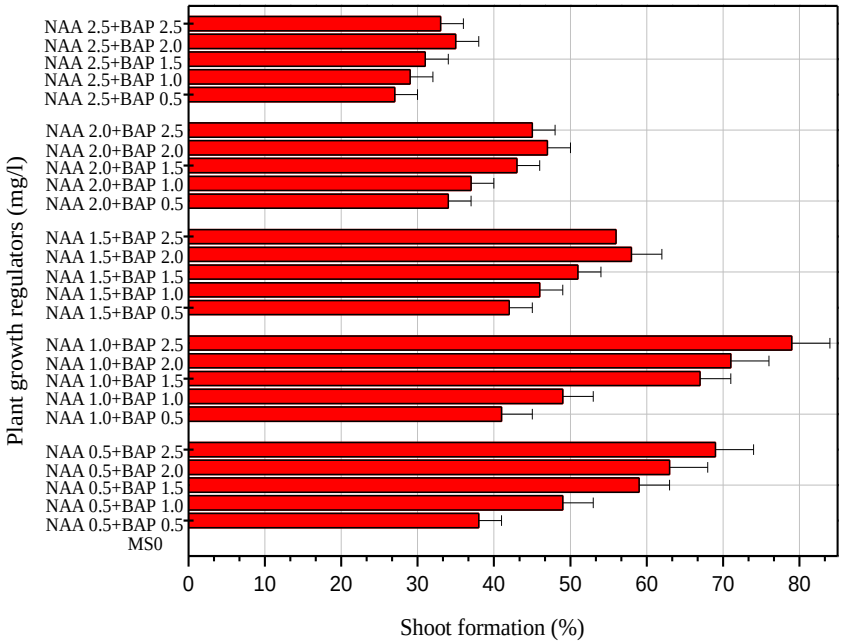

Figure 6b: Indirect shooting frequency at combination of NAA and BAP.

To get maximum number of shoots, regenerated shoots were further sub cultured on MS media having similar PGRs concentration and number of shoots per explant was recorded by visual observations (Fig. 7a,b). TDZ and NAA in combination induced optimum number of shoots per explant. At concentration of 2.5 mg/l + 1.0 mg/l TDZ and NAA maximum number of shooting was observed i.e. 12 shoots per explant. However, the lowest (2 shoots) number of shoots per explant was recorded on NAA 2.5 mg/l in combination with BAP 0.5 mg/l (Fig. 7b). TDZ 1.5 mg/l alone showed optimum response to shoot proliferation while with further increase an inhibitory effect was observed (Fig. 7a). With augmentation of NAA 1.0 mg/l the TDZ effect of shoot proliferation was increased but on the other hand reduction in number of shoots per explant was observed with increase of NAA concentration from 1.0 mg/l to 2.5 mg/l (Fig. 7a).

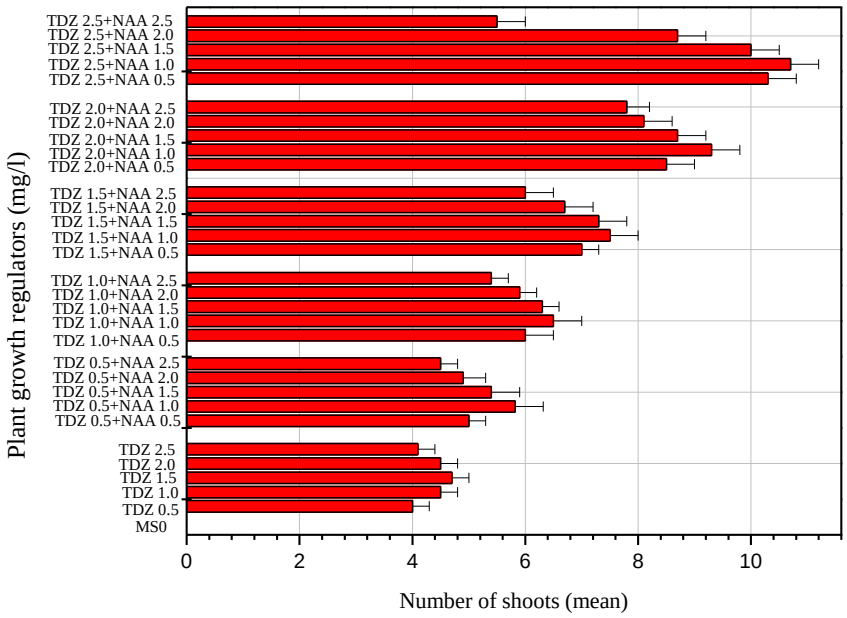

Figure 7a: Number of shoots (mean) at TDZ alone and in combination with NAA.

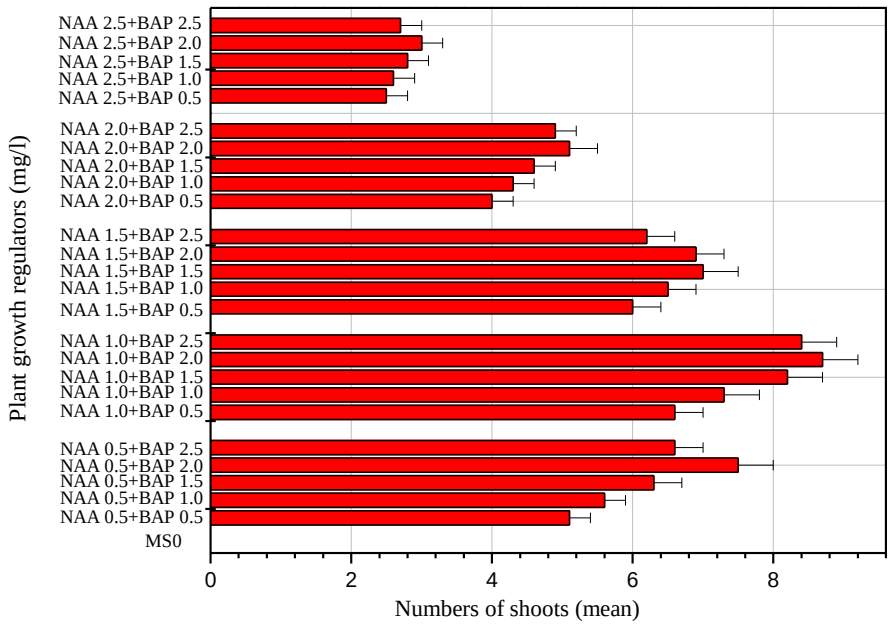

Figure 7b: Number of shoots (mean) at combination of NAA and BAP.

NAA in combination with BAP was tested for shoot proliferation (Fig. 6b). No shoot proliferation was observed on MS0. Initially the shoot proliferation efficiency was higher i.e. when NAA (0.5 and 1.0 mg/l) was used but a significant reduction in number of shoots per explant was observed with increase in NAA concentration.

After shoot induction and multiplication, shoots were transferred to shoot elongation medium. The shoot induction, multiplication and elongation medium had same concentrations of plant growth regulators. The mean shoot length per explant was recorded in range of 2 to 6cm (Fig. 9 a, b). Mean shoot length from highest to lowest was found sequentially on TDZ+NAA, BAP+NAA and on TDZ alone.

Figure 8**:** Shoot elongation in *Brassica oleracea* var. *acephala*

MS0 was found to have no effect on shoot elongation while TDZ (0.5, 1.0, 1.5, 2 and 2.5 mg/l) alone showed a moderate response and the highest shoot length 3.8 cm was recorded for 1.5 mg/l TDZ; however, further increase in TDZ concentration to 2.5 mg/l showed a negative effect on mean shoot length (Fig. 9a). TDZ in combination with NAA showed an increase in mean shoot length which was significantly higher than that found on NAA in combination of BAP. The optimum mean shoot length 5.4 cm was obtained on TDZ 2.5 mg/l and NAA 1.0 mg/l. Similarly, optimum mean shoot length of 5 cm was recorded with NAA 0.5 mg/l +BAP (2.5 mg/l) but reduction in length was observed with increase of NAA concentration (Fig. 9b).

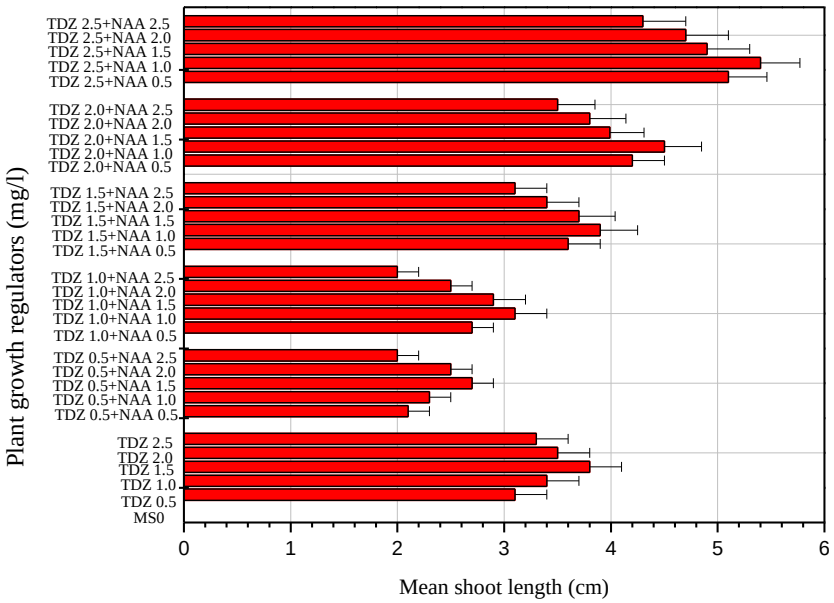

Figure 9a**:** Mean shoot length (cm) of regenerated shoots on TDZ alone and with NAA

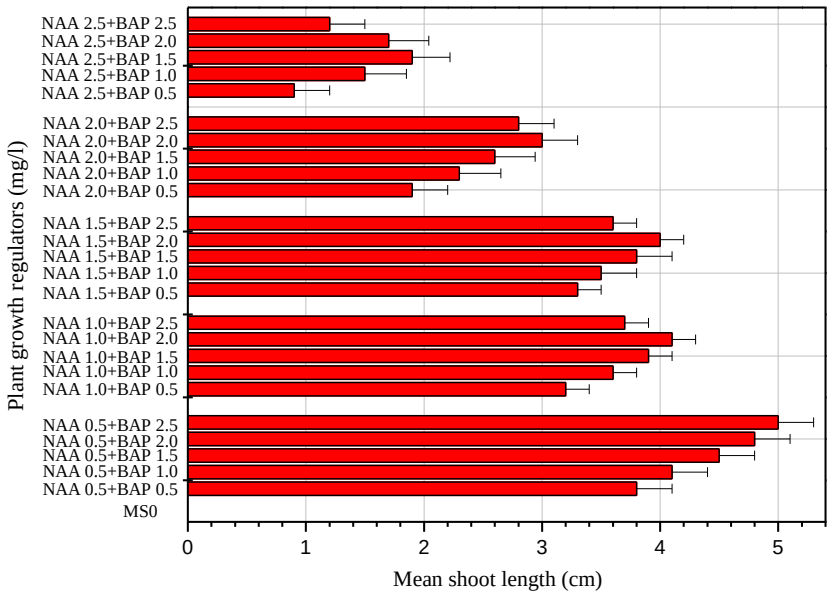

Figure 9b: Mean shoot length (cm) of regenerated shoots on combination of NAA and BAP

4.2.3 Rooting

Elongated shoots were transferred to MS media supplemented with different concentrations of NAA, IBA, IAA, MS0 and 1/2 strength MS medium. Slightly visible root outgrowths were observed on IBA (0.1 mg/l) while maximum viable rooting was observed on NAA (1.0 mg/l).

4.3 Adventitious root culture

4.3.1 Induction of adventitious roots

Three different explants (leaf, stem and root) were comparatively tested for best response to produce adventitious roots and leaf explant was selected with highest potential for adventitious rooting. Leaf explant was excised from 7-day old *in vitro* seed derived plantlet and inoculated aseptically on MS0 (as a control) and on MS medium supplemented with different auxins (NAA, IAA, IBA) in varying concentrations. The induction efficiency of auxins was determined as percent root induced per treatment, mean number of roots, Fresh weight (FW) in grams (g) and Dry weight (DW) (Table 2).

Leaf explant inoculated on PGRs free Media showed no rooting, while on media supplemented with NAA, IAA and IBA, whitish threads of adventitious roots were observed at 10^{th} day of inoculation. NAA was found as the best auxin that induced maximum adventitious roots of maximum FW as well DW from leaf explant (Fig. 10). The highest rooting frequency (87 %) and 35 roots per explant of highest FW (2.98 g) and DW (0.238 g) was recorded at MS media supplemented with NAA (1.0 mg/l) followed by 80 % and 25 roots per explant of 2.78 g FW and 0.222 DW at 0.5 mg/l, while further increase the NAA concentration 1.5 mg/l a mild inhibitory effect was observed and reduction in Fresh weight, number of roots per explant and in rooting frequency was also observed. Comparative to IBA, IAA showed the optimum frequency (70%) of adventitious rooting and fresh weight (FW) of 1.239 g per explant at 1.5 mg/l while 0.1 mg/l showed no effect. The adventitious root induced by IAA and IBA were slender, shorter in length and lowest biomass (FW and DW), comparative to long thick roots induced by NAA. 2.5mg/l IBA was the optimum concentration that induced 3 roots per explant with frequency of 56 %, FW and DW of 1.757 g and 0.097 g respectively.

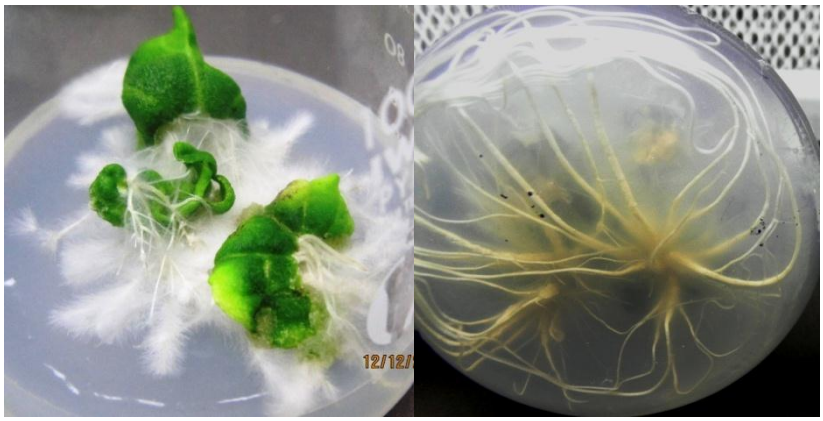

Figure 10: Adventitious root induction and elongation

Table 2: Induction of Adventitious roots from leaf explant at different concentration of auxins

S. No	Auxin	Concentration	% rooting	Mean Number of roots/explant	FW (g) /Explant	Dry weight (g)
1.	MS0	0	0	0	0	0
2.	NAA	0.1 mg/l	70 %	15	1.98	0.157
		0.5 mg/l	80 %	25	2.78	0.222
		1.0 mg/l	87 %	35	2.986	0.238
		1.5 mg/l	77 %	27	2.38	0.190
3.	IAA	0.1 mg/l	0	0	0	0
		0.5 mg/l	20 %	2	1.056	0.084
		1.0 mg/l	33 %	4	1.137	0.090
		1.5 mg/l	70 %	7	1.239	0.091
4.	IBA	0.1 mg/l	0	0	0	
		0.5 mg/l	27 %	1	1.052	0.084
		1.0 mg/l	36 %	1	1.054	0.086
		1.5 mg/l	56 %	3	1.157	0.097

Induction of adventitious root was also studied at varying pH and different sucrose concentrations. These two parameters plays important role in root induction. To find out the effects of pH and sucrose concentration, media was supplemented with a constant concentration of NAA (1.0 gm/L) and response to these parameters was record in term of percent rooting, fresh weight (FW) and dry weight (DW) as shown in Table 3 and 4 respectively.

High variation in rooting efficiency was observed at varying pH of the media supplemented with NAA 1.0 gm/l. The optimum pH for adventitious root induction from leaf explant was 5.8 in terms of 85.5% rooting, 2.53 g fresh weight and 0.186 g dry weight (Table 3). It was observed that the lowest rooting frequencies of 53.6% and 23% were observed on both pH extremes i.e. acidic (4) and basic (8), respectively.

Table 3: Effect of pH on percent rooting, FW (Fresh weight in gram) and DW (Dry weight in grams)

S. No	pH	% rooting	FW (g)	DW (g)
1.	4	53.6 %	0.93	0.077
2.	5	71 %	1.21	0.100
3.	5.8	85.5 %	2.53	0.186
5.	7.0	47 %	0.75	0.061
6.	8	23 %	0.23	0.018

Apart from pH carbon source plays a vital role in root induction and effects growth and development of the plants. For studying the effect of sucrose concentration leaf explants were cultured on MS medium supplemented with NAA 1.0 mg/l and varying concentration of sucrose (0, 1%, 2%, 3%, 4%, 5% and 6%). No rooting was found on MS medium without sucrose (0%). While varying degrees of rooting was observed on medium containing 1%, 2%, 3%, 4%, 5% and 6% sucrose. An initial increase in adventitious rooting from 81.6% to 93% on 1%, 2% and 3% sucrose and than a decline from 93% to 71% was observed on 4%, 5% and 6% was observed (Table 3). The optimum sucrose concentration that yielded maximum percent rooting (93 %), maximum FW (2.23 g) and DW (0.1 g) was observed at 3 %sucrose concentration while the

minimum percent rooting (71 %), FW (0.53 g) and DW (0.043 g) was found on higher, 6 % sucrose concentration (Table 4).

Table 4: Effect of sucrose concentration on adventitious rooting in term of percent rooting fresh weight and dry weight

S. No	Sucrose conc. (w/v)	% rooting	FW (g)	DW (g)
1.	0 %	0	0	0
2.	1 %	81.6 %	0.87	0.055
3.	2 %	89.4 %	1.98	0.080
5.	3 %	93%	2.23	0.100
6.	4 %	86.7 %	1.43	0.058
7.	5 %	79.3 %	0.64	0.052
8,	6 %	71 %	0.53	0.043

4.3.2 Maintenance of adventitious root culture

The adventitious roots were excised from the leaf explant and were transferred to shake flask containing MS liquid medium supplemented with NAA 1.0 mg/l. Before going to growth kinetics study the optimum inoculums size, 0.5 g/30ml was selected. The liquid media supplemented with NAA 1.0 mg/l was inoculated with 0.5 g inoculums and was incubated in shaker incubator. Sampling after each week up to 56 days was made and fresh weight and dry weight was recorded. From these two parameters growth ratio was elucidated (Table 5). Fig 11 shows different step of adventitious root culture.

Figure 11: *B oleracea* var *acephala* adventitious root culture

Table 5: Growth parameters with time (weeks)

S. No	Weeks	FW	DW	Growth ratio
1	0	0.5	0.06	0
2	1	0.716	0.086	0.433
3	2	1.362	0.163	1.71
4	3	1.8317	0.219	2.65
5	4	2.4103	0.289	3.816
6	5	2.8423	0.3424	4.706
7	6	3.1843	0.377	5.283
8	7	2.8909	0.342	4.7
9	8	2.7483	0.325	4.41

FW: Fresh weight, DW: Dry weight

The maximum FW (3.184 g), DW (0.377 g) and growth ratio (5.28) was observed at 6th week (42 days). And a slight decline in FW (2.89 g), DW (0.342 g) and growth ratio (4.7) was found at 7th week. The increase in dry biomass was correlated with decrease in Electrical conductivity (Fig. 10). With passage of time EC and TSS (total suspended solids or %Brix) decreased from 4.46 µs and 2.9 %Brix respectively (Table 5).

Table 6: Electrical conductivity and total suspended solids in the used media

S. No	Week(s)	Conductance (µS)	Temperature	TSS (%Brix)
1	1	4.46	26.2	**2.9**
2	2	4.48	26.2	**2.7**
3	3	4.88	26.3	**2.7**
4	4	4.56	26.3	**2.5**
5	5	3.92	26.1	**2.3**
6	6	3.39	26.2	**1.9**
7	**7**	**3.13**	**26.2**	**1.6**

TSS: Total suspended solids

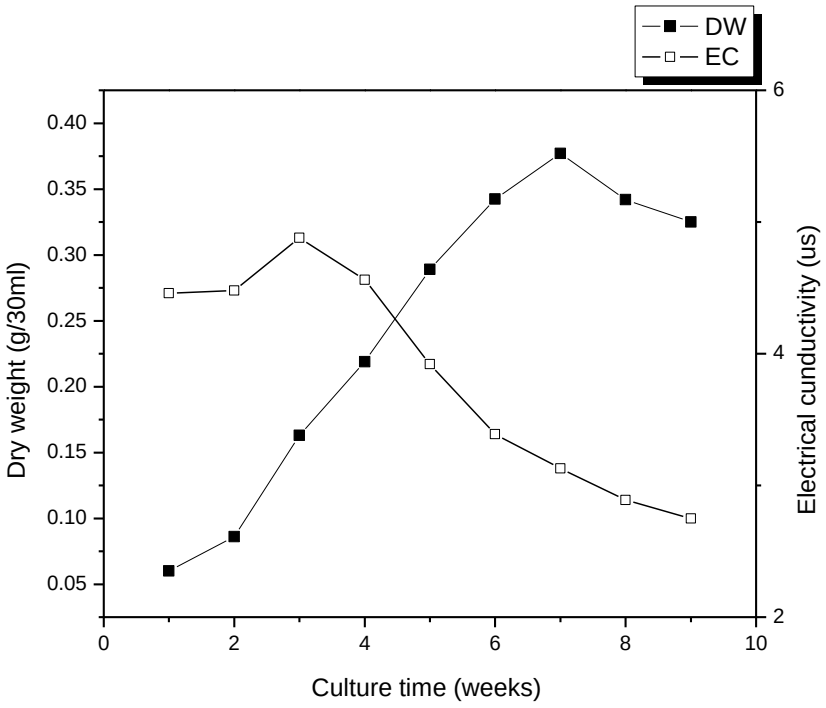

Figure 12: Changes in EC and DW of adventitious root culture in 100 ml shake flask

EC: Electrical conductivity, DW: Dry weight

4.4 DPPH Free Radical Scavenging Assay

Methanol extract of regenerated tissues was obtained and were analysed for DPPH free radical scavenging activity. The highest 36.58 % radical scavenging activity was observed in seed derived plant lets and the lowest 7.8 % was observed in adventitious roots directly excised from leaf explant. Among the regenerated tissues, regenerated plantlets and adventitious roots 2 showed a higher activity than callus. The results showed that differentiated tissues had a higher antioxidant activity than that in dedifferentiated tissue in this plant.

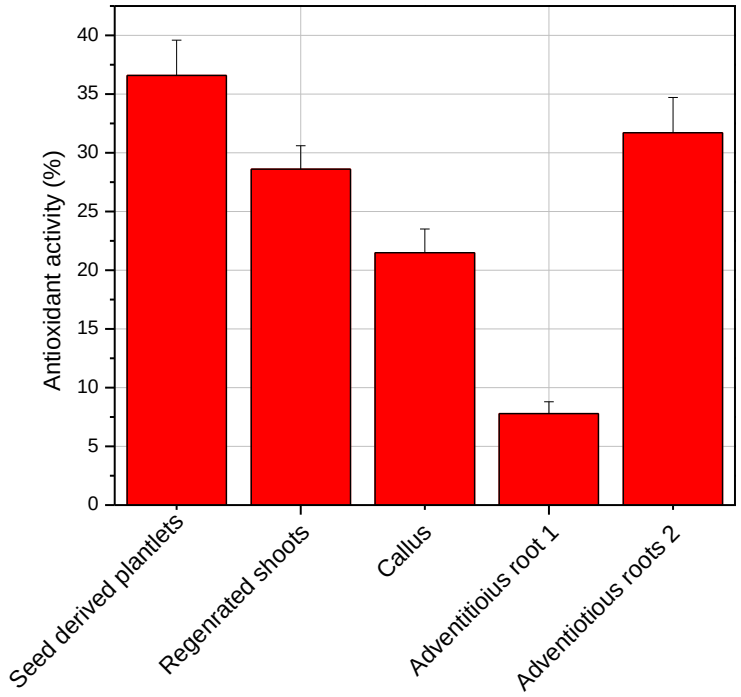

Figure 13: Antioxidant activity of regenerated tissues

Adventitious root 1: excised from leaf explant,

Adventitious root 2: roots sub cultured in Liquid medium

5. DISCUSSION

The overall objective of present study was to find out the morphogenic potential of TDZ, adventitious root culture and to evaluate anti oxidant activity of different regenerated tissues of *Brassica oleracea* var *acephala*. Thiadiazuron, phenyl-urea is an effective synthetic herbicide that shows potent cytokinin like activity (Mok *et al.,* 1982). TDZ was classified as a type of cytokinin that induces many responses that were similar to the responses induced by natural cytokinins. It was proved that TDZ, unlike traditional phytohormones, individual fulfilled the requirements of various regenerative responses of many different plant species (Guo *et al.,* 2011). A range of cytokinins (Kinetin, BA, 2-iP and zeatin) has also been used in micropropagation research (Bhojwani and Razdan, 1992). But a wider survey of the existing literature suggests that BA is the most reliable and useful cytokinin. A number of plants have been successfully multiplied on medium containing BA.

Several studies have been made by researchers on *In vitro* regeneration of *Brassica* species, including *B. oleracea* var. *italica* (Huang *et al.,* 2011; Kim *et al.,* 2002; Ravanfer *et al.,* 2009); *B. oleracea* var. *capitata* (Munshi *et al.,* 2007); *B. oleracea* var. *acephala* (Peng-fang *et al.,* 2003; Dai *et al.,* 2009) and Pavlovic *et al.* (2010) studied regeneration potential of four different cabbage cultivars (red cabbage, Broccoli, savoy cabbage and cauliflower). Christey and Earle (1991) studied regeneration of five *B oleracea* species from peduncle explants.

The leaf explant used in current study responded to all PGRs with varying intensity. The best (85%) callus induction was recorded on MS medium containing BAP (1.5 mg/l) in combination with NAA (1 mg/l) than MS medium containing TDZ (2.5 mg/l) in combination with NAA (1 mg/l) which was 82%. NAA alone were also applied but direct adventitious root induction was observed and in combination with TDZ and BAP synergistic effect on callogenesis was observed. Lowest callus formation frequency was recorded on TDZ (0.5) alone and a mild increase in callus frequency was observed with increase in TDZ concentration. Similar values have been reported for *B. rapa* var. *turnip* (Abbasi *et al.,* 2010). While Peng-fang *et al.* (2003) reports slightly different concentrations of BAP in combination with NAA, they reported 4 mg/l BAP and 0.1 mg/l NAA was the optimum concentration for callus induction.

The highest indirect shooting (85%) was recorded on TDZ 2.0 mg/l in combination with NAA 0.5 mg/l followed by 83% on NAA 0.5 mg/l in combination with BAP 2.5 mg/l. The lowest shooting frequency 37% was observed on TDZ 0.5 mg/l. Ours findings are similar to Peng-fang *et al.* (2003), they reported MS medium containing BAP 2mg/l in combination with NAA 0.02 mg/l was the best regeneration media for *B oleracea* var *acephala*. While Dai *et al.* (2009) used cotyledon and hypocotyls explants of *B oleracea* var *acephala* (ornamental kale) and observed 76.1% and 65% direct shoot induction from Hypocotyl and cotyledon explants respectively on MS medium containing BAP 3 mg/l + 0.1 mg/l NAA. They concluded that the best responsive explant to regeneration was hypocotyl. Cheng *et al.* (2001) also reported more than 90% regeneration using 4.5 mg/l BA and 94% with 0.5 mg/l TDZ in combination with IAA 0.01 mg/l from 3 day old hypocotyl explant of *B. oleracea*. Contrary to present study Jonoubi *et al.* (2004) reported maximum regeneration frequency from hypocotyl explant of *B. napus* L. at MS medium containing BAP 4.5 mg/l and TDZ 0.3 mg/l from callus, induced at 2,4-D 1 mg/l. Aoun *et al.* (2008) used transverse thin cell layer explant of *B juncea* (L.) Czern. and obtained maximum shooting frequency at MS medium supplemented with BAP 53.3 µM (12 mg/l) in combination with NAA 3.22 µM (0.596 mg/l). Ahmad *et al.* (2010) reported that combination of BAP and NAA inhibited shooting in *Piper nigrum* which is contrary to our results of highest frequency of shooting at BAP+NAA as compared to other PGRs tested. The contradictions in ours findings to previous reports might be due to specie and genotype differences. Murata and Orton (1987) reports specie dependant variations in callus formation and shooting frequencies to PGRs concentrations, they studied seven *Brassica* species for re and de-differentiation and found varying frequency of callus formation and shoot formation.

The regeneration potential was determined in term of mean shoot number and mean shoot length (cm). The maximum number (12) of shoot per explant was observed at MS medium supplemented with TDZ 2.5 mg/l and NAA 1 mg/l, which is similar to findings of Cheng *et al.* (2001), they obtained maximum number (8.5) shoots per cotyledon explant of *B oleracea* on MS medium containing IAA0.5 µM and TDZ 2.5 µM. But contrary to ours finding he observed high proliferative affect of BAP alone than TDZ in combination with IAA. Dai *et al.* (2009) obtained 4.3 shoots per cotyledon explant and 8.2 from hypocotyl explant. Ravanfer *et al.* (2011) reported 0.43 shoots per cotyledon explant on MS medium containing 3 mg/l BAP and 1 mg/l NAA. Results clearly shows

that increasing NAA concentration shoot formation frequency as well as number of shoots decreases while increasing TDZ or BAP concentration shoot formation frequency increased and shows cytokinine like activity of TDZ.

Adventitious roots that arise from non pericyclic tissues in aged roots, leaves and stems also known as post embryonic root. It plays important role in vegetative propagation. There are many endogenous and exogenous factors/mediators that switch the adventitious root formation. These factors are Ca^{++}, auxin, nitric oxide, carbon monoxide, sugars, ethylene, polyamines, peroxides, etc. Out of these auxin plays a key role in adventitious root formation. There are two pathways for adventitious root formation, direct organogenesis (from cells types like cambium) and from callus after mechanical cuttings (Li *et al.,* 2009). For obtaining higher biomass, adventitious root culture is more efficient than other plant tissues due to higher growth rate and another important feature is consistent metabolite productivity (Murthy *et al.,* 2008). To achieve high biomass optimization of Invetro culture condition is important. In this study we optimized these conditions and obtained higher biomass of adventitious root and were assessed for DPPH.

In induction phase leaf explant were inoculated at MS medium containing different concentrations of Auxins (NAA, IBA, IAA). The best results in term of % roots induced, number of roots, fresh biomass and dry biomass was achieved at MS medium containing 1.0 mg/l NAA. Which make a contradiction to findings of Wu *et al* (2006), because he used *Echinacea angustifolia* and achieved best root proliferation at MS medium containing 2 mg/l IBA. Ours experiment also depict that with increase in NAA concentration reduction in adventitious root was observed. Goel *et al* (2008) induced maximum adventitious root from callus of *Hypericum perforatum* at MS medium containing 4.0 mg/l IAA. In ours study for both IAA and IBA significantly lower induction response was observed that might be due to their lower stability as IAA is more readily photo oxidized (Nissen and Sutter 1990). It also depends on affinity of these auxins to auxin receptor protein which is involved in rooting (De Klerk *et al.,* 1999). Similar to ours finding Zhu *et al.* (2010) reported best induction from leaf explant of *B. oleracea* and *B. juncea* chimeras at MS medium containing NAA as root inducing PGR. Similar to our finding; Karin *et al.* (2005) also reported NAA as best auxin for Mung bean.

Sucrose an important source of carbon, that regulates osmotic pressure in medium (Calamar and De Klerk, 2002). Several reports have shown effects of sucrose concentration in medium on adventitious root induction (Hussein *et al.,* 2011; Takahashi *et al.,* 2003). No root induction was observed at MS0 (3.0 % sucrose and free of PGRs). While finding out the effect of sucrose MS medium was supplemented with 1.0 mg/l NAA and sucrose was added in varying concentrations (1, 2, 3, 4, 5 and 6 %). MS medium without sucrose (0 %) was considered as control. Best root induction (highest rooting frequency, fresh and dry biomass as well as number of roots per explant) was observed at 3 % sucrose while further increasing or decreasing the sucrose concentration reduction in adventitious root was observed. These are similar to findings of Cheng *et al,* (1992), they reported best rooting in *Eucalyptus sideroxylon* at sucrose concentration in range of 2-3%. Rooting in *Panax ginseng* was limited at higher sucrose concentration while it was remarkably increased in range of 1-3 % (Jung *et al.,* 2005). Pierik and Steegmans (2003) concluded from their study on *Rhododendron,* that Auxins are absolutely required for adventitious root induction and sugar also plays an important role while pH and boric acid did not.

After induction roots were excised and cultured in shake flask to study growth kinetics of growing roots in 56 days (8 weeks) time frame. It showed a lag phase of 5days and than an increase in growth in term of fresh biomass and dry biomass was observed, which is similar to hairy growth rate of *Artemisia annua* (Liu *et al.*, 1997). In parallel to biomass electrical conductivity of medium decreased, which confirms uptake of nutrients from medium and increase in biomass.

Brassica vegetables have higher potential of free radical scavenging which make it very important to the consumer point of view (Soengas *et al.,* 2011). The antioxidant potential of *Brassica* vegetables is due sulforaphane (Sivakumar *et al.*, 2007) and phenolic compounds (Cartea *et al.*, 2011). In the present study, *in vitro* antioxidant activity of seed derived and *in vitro* regenerated tissues was determined. The results showed that differentiated tissues had a higher antioxidant activity than callus. Previously, abietane diterpene antioxidants were found only in shoot cultures of *S. officinalis* (Grzegorczyk *et al.,* 2007) and rosemary (Caruso *et al.,* 2000) and not in callus, suspension or hairy roots. Recently, Ahmad *et al.,* (2010) also reported a lower antioxidant activity in callus cultures of *Piper nigrum* as compared to regenerated shoots and plantlets which is contrary to present study. Similarly, Kintzios *et al.* (2004) found that the accumulation of

rosmarinic acid in *O. basilicum* cell cultures was markedly lower than in regenerated plantlets. All these results suggested that in some cases differentiated tissues are superior in accumulation of secondary metabolites as compare to undifferentiated tissues. On the other hand, comparison of antioxidant activity between regenerated and seed derived tissues showed a comparative activity in seed derived shoots than that of regenerated tissues. According to Matkowski (2008), in some cases full development in natural conditions is useful for producing a considerable amount of secondary products and it has been reported for several classes of metabolites, especially for alkaloids, and has also been published for some antioxidant compounds. Our results are closely related with the findings of Thiem and Krawczyk (2003) who found that ellagic acids present in *Rubus chamaemorus* plants was over 10 times lower in callus and 3 times lower in shoot cultures.

6. REFERENCES

Abbasi BH, Khan MA, Mahmood T, Ahmad M, Chaudhary MF (2010) Shoot regeneration and free-radical scavenging activity in *Silybum marianum* L. Plt Cell Tiss Org cult101: 371-376.

Abbasi BH, Saxena Pk, Murch SJ, Liu CZ (2007). *Echinacea* biotechnology: Challenges and apportunities. *In vitro* Cell Dev Biol Plant 43: 481-492.

Ahmad N, Fazal H, Abbasi BH, Rashid M, Mahmood T, Fatima N (2010) Efficient regeneration and antioxidant potential in regenerated tissues of *Piper nigrum* L. Plant Cell Tiss Organ Cult 102: 129-134.

Akashia T, Ishizakia M, Aoki T, Ayabe S (2005) Isoflavonoid production by adventitious-root cultures of *Iris germanica* (Iridaceae). Plant Biotech 22: 207-215.

Almeida D, Rosa E (1994) Protein and mineral concentration of Portuguese Kale *(Brassica oleraceae* var. *acephala)* related to soil composition, ISHS *Brassica* Symposium - IX Crucifer Genetics Workshop.

Aoun M, Charles G, Hourmant A (2008) Micropropagation of three genotypes of Indian mustard [{Brassica juncea} (L.) Czern.] using seedling-derived transverse thin cell layer (tTCL) explants. African J of Biotech 9: 2621-2626.

Arnison PG, Donaldson P, Jackson A, Semple C, Keller W (1990) Genotype-specific response of cultured broccoli (*Brassica oleracea* var. *italica*) anthers to cytokinins. Plant Cell Tissue Organ Cul 20: 217-228.

Art ICW, Hollmam PCH (2005) Polyphenols and disease risk in epidemiologic studies. American J of Clinc Nutri 81: 317S-325.

Arteel GE (2003) Oxidants and antioxidants in alcohol induced liver disease. Gastroenterol 124:778-790.

Balkaya A, Yanmaz R, Apaydin A, Kar H (2005) Morphological characterisation of White head cabbage (*Brassica oleracea* var. *capitata* subvar. *alba*) genotypes in Turkey. New Zealand J of Crop & Hort Sci 33: 333-341.

Barlow PW (1986) Adventitious root culture of whole plants: their Forms, Function and Evolution. In new root formation in plants and cuttings. MB. Jakson Dordrecht, Netherland. Maartinus Nijhoff Publishers pp. 67-80.

Barros L, Ferreira MJ, Queiros B, Ferreira ISCF, Baptista P (2007) Total phenols, ascorbic acid, β-carotene and lycopene in Portuguese wild edible mushrooms and their antioxidant activities. Food Chem 103: 413-419.

Belehu T, Hammes PS, Robbertse PJ (2004) The origin and structure of adventitious roots in sweet potato (Ipomoea batatas). Aust Journal 45: 94-101.

Bergmann F (1970) The biosynthesis of glucosinolates in the course of ontogenesis of *Sinapis alba* L. Plt Physiol 62: 362-375.

Bhalla PL, Weerd N (1999) *In vitro* propagation of cauliflower, *Brassica oleracea* var. *Botrytisfor* hybrid seed production. Plnt Cell Tissue & Org Cult 56: 89-95.

Bhojwani SS, Razdan MK (1992) Plant tissue culture: Theory and practice, Elsevier, Amsterdam, London, New York, Tokyo pp. 203-209.

Blakesley D (1994) Auxin metabolism and adventitious root initiation. In Biology of Adventitious Root Formation. Eds. T.D. Davis and B.E. Haissig. Plenum Press, New York, pp. 143-154.

Blois MS (1958) Antioxidant determinations by the use of a stable free radical. Nature 181: 1199-1150.

Bolton JL, Trush MA, Penning TM, Dryhurst G, Monks TJ (2000) Role of quinones in toxicology. Chem Res Toxicol 13:135-160.

Borisjuk L, Walenta S, Weber H, Mueller-Klieser W, Wobus U (1998). High resolution histographical mapping of glucose concentration in developing cotyledon of *Vicia faba* in relation to mitotic activity and storage process: glucose as a possible development trigger. Plt J 15: 583-591.

Borkowski J, Szajdek A, Borkowska EJ, Ciska E, Zielinski H (2008) Content of selected bioactive components and anti oxidant properties of broccoli (*Brassica oleracea L.*). European Food Res & Technol 226: 459-465.

Cabbage- common (*Brassica oleracea* L.var. *acephala*), callus deriving from leaves

Calamar A, De Klerk GJ (2002) Effect of sucrose on adventitious root regeneration in apple. Plant Cell Tiss Organ Cult 70: 207-212.

Caldero´n Baltierra X, Montenegro G, De Garcia E (2004) Ontogeny of in vitro rooting processes in *Eucalyptus globulus*. Plant Cell Rep 18: 89-96.

Cao J, Earle ED (2003) Transgene expression in broccoli (*Brassica oleracea* var. *italica*) clones propagated *in vitro* via leaf explants. Plant Cell Rep 21: 789-796.

Cartea ME, Francisco M, Soengas P, Velasco P (2011) Phenolic Compounds in *Brassica* Vegetables. Molecules 16: 251-280.

Cartea ME, Velasco P, Obrego´ n S, Padilla G, de Haro A (2008) Seasonal variation in glucosinolate content in Brassica oleracea crops grown in northwestern Spain. Phytochem 69: 403-410.

Caruso JL, Callahan J, DeChant C, Jayasimhulu K, Winget GD (2000) Carnosic acid in green callus and regenerated shoots of *Rosmarinus officinalis*. Plant Cell Rep 19: 500-3.

Carvalho AM (2010) Plantasy sabiduría popular del Parque Natural de Montesinho. Un estudio etnobotánico en Portugal. Biblioteca de Ciencias 35. Madrid: Consejo Superior de Investigaciones Científicas.

Carvalho EB, Curtis WR (1998) Characterization of fluid flow resistance in root cultures with a convective flow tubular bioreactor. Biotechnol Bioeng 60: 375–384.

Chand H, Pearson MN, Lovell PH (1999) Rapid vegetative multiplication in *Colocasia esculenta* (L) Schott (taro). Plant Cell Tiss Org Cult 55: 223-226.

Cheng B, Peterson CM, Mitchell RJ (1992). The role of sucrose, auxin and explant source on *in vitro* rooting of seedling explants of *Eucalyptus sideroxylon*. Plant Science, 87: 207-214

Cheng C, Lakshmanan P, Swarup S (2001) High frequency direct shoot regeneration and continuous production of rapid-cycling *Brassica oleracea* in vitro. *In vitro* cell Dev Biol Plant 37: 592-598.

Christensen S, von Bothmer R, Poulsen G, Maggioni L, Phillip M, Andersen BA, Jørgensen RB (2011) AFLP analysis of genetic diversity in leafy kale (*Brassica oleracea* L. convar. *acephala* (DC.) Alef.) Landraces, cultivars and wild populations in Europe. Genet Resour Crop Evol 58: 657-666.

Christey MC and Earle ED (1991) Regeneration of *Brassica oleracea* from Peduncle Explants. Hortscience 26: 1069-1072.

Cogbill S, Faulcon T, Jones G, McDaniel M, Harmon G, Blackmon R, Young M (2010) Adventitious shoot regeneration from cotyle- 400 donary explants of rapid-cycling fast plants of Brassica rapa L. Plant Cell Tissue Organ Culture 101: 127-133.

Conaway CC, Getachun SM, Liebes LL, Pusateri DJ, Tophan DKW, Botero-Omary M, Chung FL (2001) Disposition of glucosinolates and sulphoraphanes in human after ingestion of steam and fresh broccoli. Nutrition and Cancer 38: 168-178.

Cosgrove DJ (1999) Enzymes and other agents that enhance cell wall extensibility, Annual Review of Plant Physiology and Plant Molecular Biology 50: 391-417.

Crozier A, Jaganath IB, Clifford MN (2006) Phenols, polyphenols and tannins: An overview. In *Plant Secondary Metabolites: Occurrence, Structure and Role in the Human Diet*; Crozier, A., Clifford, M., Ashihara, H., Eds.; Blackwell: Oxford, UK, 2006; pp. 1 24.

Dai XG, Shi Xp, Ye Ym, Fu Q, Bao MZ (2006) High frequency plant regeneration from cotyledon and hypocotyl explants of ornamental kale. Biologia Planta 53: 769-773.

Dai XG, Shi XP, Ye YM, Fu Q, Bao MZ (2009) High frequency plant regeneration from cotyledon and hypocotyl explants of ornamental kale. Biologia Plantarum 53: 769 - 773.

De Klerk GJ (1995) Hormone requirements during the successive phases of rooting of Malus microcuttings. In: Terzi, M.; Cella, R.; Falavigna, A., eds. Current issues in plant molecular and biology. Dordrecht: Kluwer Academic Publishers; 1995:111-116.

De Klerk GJ, Krieken WVD, Jong J (1999) The formation of adventitious roots: new concepts, new possibilities. *In vitro* Cell Dev Biol 35: 189-199.

Demir E, Balkaya A (2005) Seed development stages of kale (Brassica oleracea var. acephala L.) genotypes in Turkey. Hort Sci (Prague) 32: 147-153.

Dikson MH, Wallance DH (1986) "Cabbage Breeding" In breeding vegetable crops. Edited by Mark J. Bassett, westport, com.; AVI publishing Company pp. 39-538.

Dobranszki J, da Silva JA (2010) Micropropagation of apple-A review. Biotech Advances 28: 462-488.

Dobránszki J, da Silva JA (2010) Micropropagation of apple-A review. Biotechnology Advances 28: 462-88.

Donato VMTS, de Andrade AG, Cabral JB (2002) *In vitro* regeneration of plants of tissue. Rev Ecossistema 26: 143-146.

Drew RA (1988) Rapid clonal propagation of papaya in vitro from mature field grown tree. Hort Sci 23: 143-146.

Duke J (1983) Handbook of Energy Crops Published only on the Internet, excellent information on a wide range of plants. pp. 78.

Eapen S, Tivarekar S, George L (1998) Thidiazuron-induced shoot regeneration in pigeonpea (*Cajanus cajan* L.). Plant Cell Tiss and Org Cult 53: 217-220.

Esau K (1977) Anatomy of seeds plants. (2^{nd} edition) John Wiley Publisher.New York pp. 213-219.

Fabre N, Poinsot V, Debrauwer L, Vigor C, Tulliez J, Fouraste I, Moulis C (2007) Characterization of glucosinolates using electro spray ion trap and electro spray quadrupole time of flight mass. Phytochemical Analysis 18: 306-319.

Fahey JW, Wade KL, Stephenson KK, Chou FE, (2003) Separation and purification of glucosinolates from crude plant homogenates by high-speed counter-current chromatography. J Chromato Analys 996: 85-93.

Falasca G, Altamura MM (2003) Histological analysis of adventitious rooting in *Arabidopsis thaliana* (L.) Heynh seedlings. Plant Cell Envir 137: 441-448.

Falasca G, Zaghi D, Possenti M, Altamura MM (2004) Adventitious root formation in *Arabidopsis thaliana* thin cell layers. Plant Cell Rep 23: 17-25.

Femina A, Robertson JA, Waldron KW (1998) Cauliflower (*Brassica oleracea L.*), globe antichoke (*Cynara scolymous L.*) and Chicory witloof (*Cichorium intybus*) processing by-products as source of dietary fibre. J of the Sci of Food & Agri 77: 511-518.

Ferreres F, Fernandes F, Sousa C, Valentao P, Pereira JA, Andrade PB (2009) Metabolic and Bioactivity Insights into *Brassica oleracea* var. *acephala*, J Agric Food Chem 57: 8884-8892.

Ferreres F, Sousa C, Valentao P, Seabra RM, Pereira JA, Andrade PB (2007) Tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC) seeds: phytochemical characterization and antioxidant potential. Food Chem 101: 549-558.

Ferreres F, Sousa C, Vrchovska V, Valentao P, Pereira JA, Seabra RM, Andrade PB (2006) Chemical composition and antioxidant activity of tronchuda cabbage internal leaves. Eur Food Res Technol 222: 88-98.

Ferreres F, Valentao P, Llorach R, Pinheiro C, Cardoso L, Pereira JA, Sousa C, Seabra RM, Andrade PB (2005) Phenolic compounds in external leaves of Tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC). J Agri Food Chem 53: 2901-2907.

Fett-Neto AG, Fett PS, Goulart LWV, Pasquali G, Termignoni RR, Ferreira AG (2001) Distinct effect of auxin and light on adventitious root development in *Eucalyptus saligna* and *Eucaliptus globules*. Tree Physiol 21**:** 457-464

Fimognari C, Nusse M, Cesari R, Iori R, Cantelli-Forti G, Hrelia P (2002) Growth inhibition, cell cycle arrest and apoptosis in human T-cell leukaemia by the isothiocyanates sulphoraphane. Carcinogene 23: 581-586.

Ford YY, Bonham EC, Cameron RWF, Blake PS, Judd HL, Harrison-Murray RS (2002) Adventitious rooting: examining the role of auxin in an easy- and a difficult-to-root plant. Plant Growth Reg 36: 149-159.

Ford YY, Bonham EC, Cameron RWF, Blake PS, Judd HL, Harriso n-Murray RS (2001) Adventitious rooting: examining the role of auxin in an easy- and a difficult-to-root plant. Plant Growth Regul. 36: 149-159.

Fujioka S, Sakurai A (1977) Biosynthesis and metabolism of brassinostroids. Physiologia Plantarum 100: 710-715.

George EF (2008) Plant tissue culture procedure –background. In: George EF, Hall MA, De Klerk GJ, editors. Plant Prop by Tissue Culture Dordrecht 3: 1-28.

George EF, Sherrington PD (1984). Plant propagation by tissue culture, London: Exegetics Ltd. pp 90-94.

Gieslene GF, Silva GL (2000) Antibacterial activity of plant extracts and phytochemicals on antibacterial resistance bacteria. Braz J Microbial 31: 21-30.

Goel MK, Kukreja AK, Bisht NS (2008) *In vitro* manipulations in St. John's wort (Hypericum perforatum L.) for incessant and scale up micropropagation using adventitious roots in liquid medium and assessment of clonal fidelity using RAPD analysis. Plant Cell Tiss Org Cult 96: 1-9.

Goel MK, Kukreja AK, Bisht NS (2009) *In vitro* manipulations in St. John's wort (*Hypericum perforatum* L.) for incessant and scale up micropropagation using adventitious roots in liquid medium and assessment of clonal fidelity using RAPD analysis. Plant Cell Tiss Org Cult 96: 1-9.

Grove MD, Spencer GF, Rohwedder WK, Mandava N, Worley JF, Jr JDW, Steffens GL, Flippen-Anderson JL, Carter Cook J (1979) Brassinolide, a plant growth-promoting steroid isolated from Brassica napus pollen. Nature 281: 216-217.

Grzegorczyk I, Matkowski A, Wysokinska H (2007) Antioxidant activity of extracts from *in vitro* cultures of *Salvia officinalis* L. Food Chem 104: 536-541.

Gugel RK, Falk KC (2006) Agronomic and seed quality evaluation of *Camelina sativa* in western Canada. Canada J Plt Sci 86: 1047-1058.

Guidi I, Galimberti D, Lonati S, Novembrino C, Bamonti F, Tiriticco M, Fenoglio C, Venturelli E, Baron P, Bresolin N (2006) Oxidative imbalance in patients with mild cognitive impairment and Alzheimer's disease. Neurobiol Aging 27: 262-269.

Gulcin I, Sat IG, Beydemir S, Elmastas M, Kufrevioglu OI (2004) Comparis on of antioxidant activity of clove (*Eugenia caryophylata* Thunb) buds and lavender (*Lavandula stoechas* L.). Food Chem 87: 393-400.

Guo B, Abbasi BH, Zeb A, Xu LL, Wei YH (2011) Thidiazuron: A multi-dimensional plant growth regulator. African J of Biotech 10: 8984-9000.

Halliwell B (1994) Free radicals, antioxidants and human disease: Curiosity, cause or consequence? Lancet 344: 721-724.

Halliwell B (1995) Antioxidant characterization. Methodology and mechanism. Biochem Pharmacol 49: 1341-8.

Hanelt, P. (ed) (2001) Mansfeld's encyclopedia of agricultural and horticultural crops (except ornamentals). Springer, Berlin. pp 50-63.

Harberd DJ (1972) A contribution to the cyto-taxonomy of *Brassica* (Cruciferae) and its allies. Bot J Linn Soc 65: 1-23.

Heimler D, Vignolini P, Dini MG, Vincieri, FF, Romani A (2006) Antiradical activity and polyphenol composition of local *Brassicaceae* edible varieties. Food Chem 99: 464-469.

Helm J (1963) Morpho-taxonomische Gliederung der Kultursippen von *Brassica oleracea* L. Kulturpflanze 11: 92-210.

Hemm MR, Ruegger MO, Chapple C **(** 2003).The *Arabidopsis* ref2 mutant is defective in the gene encoding CYP83A1 and shows both phenylpropanoid and glucosinolate phenotypes. Plant Cell 15: 179-194.

Hertog MGL, Kromhout D, Aravanis C, Blackburn H (1995) Flavonoid intake and long-term risk of coronary heart disease and cancer in the seven countries study. Archives of Internal Med 155: 381-386.

Hollman PCH, Hertog MGL, Katan MB (1996) Analysis and health effects of flavonoids. Food Chemi 57: 43-46.

Holobiuc I, Blindu R (2006) *In vitro* culture introduction for ex situ conservation of some rare plant species. Rom J Biol 51: 47-52.

Hosoki T, Shiraishi K, Kigo T, Ando M (1989) Transformation and regeneration of Ornamental kale (*Brassica oleracea* var. *acephala* DC) mediated by *Agrobacterium rhizogenes*. Scie Horticulturea 40: 258-266.

Howard BH (1994) Manipulating rooting potential in stock plants before collecting cuttings. In: Davis TD, Haissig BE (eds). pp 121-123.

Hu CY, Wang PJ (1983) Meristem, shoot tip and bud cultures. In Handbook of Plant cell culture: Techniques for propagation and Breeding. Vol 1 (eds 3) Evans DA, Sharp WR, Ammirato PV, Yamada Y, Macmillan Publishing Co., New York, pp. 177-277.

Huang D, Ou B, Prior RL (2005) The chemistry behind antioxidant capacity assays. J Agric Food Chem 53: 1841-1856.

Huang K, Wu Q, Lin J, Zheng J(2011) Optimization of a plant regeneration protocol for broccoli. African J of biotecn 10: 4081-4085.

Hussein S, Ling APK, Hann NGT, Ibrahimi R, Paek KY (2011) Adventitious roots induction of recalcitrant tropical woody plant, *Eurycoma longifolia.* Romanian Biotech Letters 17: 7026-7035.

Hyun DH, Hernandez JO, Mattson MP, de Cabo R (2006) The plasma membrane redox system in aging. Aging Res Rev 5: 209-220.

Jafri SMH (1973) *Brassicaeae* in Nasir E and Ali SI (Eds.) Flora of W. Pakistan No. 55: 1-308.

Johnson EJ, Hammond BR, Yeum KJ, Qin J, Wang XD, Castaneda C, Snodderly DM, Russell RM (2000). Relation among serum and tissue concentration of Lutein and Zeaxanthin and macular pigment density. American J Clinical Nutr 71: 1555-1562.

Jonoubi P, Mousavi A, Majid A, Daneshain J (2004) Improved *Brassica napus* L., Regenration from hypocotyl using Thidiazuon and Benzyladenine as cytokinin sources. Pak J Bot. 36: 321-329.

Josefsson E (1970) Pattern, content and biosynthesis of glucosinolates in some cultivated Cruciferae. Swedish Seed Association Publication pp.42.

Jules J (2009) Plant Breeding Reviews. 31. Wiley. p. 56. ISBN 978-0-470-38762-7.

Kanakis AG, Demetriou K (1993) *In vitro* shoot regeneration of globe artichoke from shoot apices treated with Thidiazuron and from mature zygotic embryos treated with cytokinins. J Hort Sci 68: 439-445.

Kanehiraa T, Takekoshib S, Nagatab H, Matsuzakia K, Kambayashic Y, Osamurab RY, Homma T (2003) A novel and potent biological antioxidant, Kinobeon A, from cell culture of safflower. Life Sci 74: 87-97.

Karin K, Maria H, Desana L (2005) Effect of Auxins and Plant Oligosaccharides on Root Formation and Elongation Growth of Mung Bean Hypocotyls. Plt Growth Reg 46: 1-9.

Kartha KK, Leung NL, Pahl K (1980) Cryopreservation of strawberry meristems and mass propagation of plantlets. J American Soc Hortic Sci 105: 481-484.

Kaur C, Kapoor HC (2001) Anti-oxidants in fruits and vegetables- the millennium`s health. Inter J of Food Sci and Tech 36: 703-725.

Kevers C, Hausman JF, Faivre-Rampant O, Evers D, Gaspar TH (1997) Hormonal control of adventitious rooting: progress and questions. J Appl Bot 71: 71-79.

Kim HJ, Botella JR (2002) Callus induction and Plant regeneration from Broccoli (*B oleracea* var. *italica*) for transformation. J of Plnt Biol 45: 177-181.

King JJ, Stimart DP (1998) Genetic analysis of variation for auxin-induced adventitious root formation among eighteen ecotypes of *Arabidopsis thaliana* L. Heynh. J. H ered. 89: 481-487.

Kinnula VL, Crapo JD (2004) Superoxide dismutases in malignant cells and human tumors. Free Rad Biol Med 36: 718-744.

Kintzios S, Kollias H, Straitouris E, Makri O (2004) Scale-up micropropagation of sweet basil (*Ocimum basilicum* L.) in an airlift bioreactor and accumulation of rosmarinic acid. Biotechnol Lett 26: 521-3.

Koleva II, Beek TAV, Linssen JPH, Groot AD, Evstatieva LN (2001) Screening of plant extracts for antioxidant activity: a comparative study on three testing methods. Phytochem Analysis 13: 8-17.

Konishi M, Sugiyama M (2003) Genetic analysis root formation with a novel series of temperatur e-sensitive mutants of *Arabidopsis thaliana*. Development 130: 5637-5647.

Kopsell DA, Kopsell DE, Dukash LE (2004) Variation in Lutein, ß-carotene and chlorophyll concentrations among Brassica oleracea cultigens and seasons. Hortsci 39: 361-364.

Kotake T, Nakagawa N, Takeda K, Sakurai N (2000) Auxin-induced elongation growth and expressions of cell wall-bound exo- and endo-beta-glucanases in barley coleoptiles. Plt & Cell Physiol 41: 1272-1278

Kurilich AC, Tsau GJ, Brown A, Howard L, Klein BP, Jeffery EH, Kushad M, Wallig MA, Juvik JA (1999) Carotene, Tocopherol, and Ascorbate Contents in Subspecies of *Brassica oleracea*. J Agric Food Chem 47: 1576-1581.

Kushad MK, Brown AF, Kurillicn AC, Juvik JA, Klein BP, Wallig MA, Jeffery EH (1999) Variation in glucosinolates in vegetable crops of *Brassica oleracea*. J of Agri and Food Chemistry 47: 1541-1548.

Lazzeri PA, Dunwell JM (1986). *In vitro* regeneration from seedling organs of *Brassica oleracea* var. *italica* Plenck cv. Green Comet. I. Effect of plant growth regulators. Ann Bot 58: 699-710.

Le Marchand L, Hankin JH, Kolonel LN, Beecher GR, Wilkens LR, Zhao LP (1993). Intake of specific carotenoids and lung cancer risk. Cancer Epidemiol. Biomarkers Prev 48: 183-187.

Leon P, Sheen J (2003) Sugar and hormones connections trend. Plant Sci 8: 110-116.

Li SW, Xue L, Xu S, Feng H, An L (2009) Mediators, Gene and Singnaling in Adventitious rooting. Bot Rev 75: 230-247.

Liu CZ, Wang YC, Ouyang F, Ye HC, Li GF (1997) Production of artemisinin by hairy root cultures of *Artemisia annua* L. Biotech Letter 19: 927-930.

Llorach R, Gil-Izquierdo A, Ferreres F, Tomas-Barberan FA (2003) HPLC-DAD-MS/MS ESI characterization of unusual highly glycosylated acylated flavonoids from cauliflower (*Brassica oleracea* L. var. *botrytis*) agro-industrial by products. J Agric Food Chem 51: 3895-3899.

Luciano DR, Daniel CP, Joseli S, Arthur GF (2004) Carbohydrate as regulatory factors on the rooting of *Eucalyptus saligna* Smith and *Eucalyptus globulus* Labill. Plt Growth Reg 45: 63-73.

Mahlstede JP, Watson DP (1952) An anatomical study of adventitious root development in stems of Vaccinium corymbosum. Bot Gaz 113: 279-285.

Malik KA, Saxena PK (1992) Thidiazuron induces high frequency shoot regeneration in intact seedlings of pea (*Pisum sativum*) chickpea (*Cicer arietinum*) and lentil (*Lens culinaris*). Aust J Plt Physiol 19: 731-740.

Malikova J, Swaczynova J, Kolar Z Strnad M (2008) Anti-cancer and anti-proliferative activity of natural brassionsteroids. Photochem 69: 418-426.

Mandal AKA, Chatterji AK, Dutta GS (1995) Direct somatic embryogenesis and plantlet regeneration from cotyledonary leaves of safflower. Plt Cell Tiss Org Cult 43: 287-290.

Martinez S, Olmos I, Carballo J, Franco I (2010) Quality parameters of *Brassica* spp. grown in northwest Spain. Int J Food Sci Technol 45: 776-783.

Matkowski A (2008) Plant *in vitro* culture for the production of antioxidants - A review. Biotech Advances 26: 548-560.

Mean KH, Mohamed S (2001) Flavonoid (Myricetin, Quercetin, Kaempferol, Luteolin, and Apigenin) Content of Edible Tropical Plants. J Agri Food Chem 49: 3106-12.

Miliauskas G, Venskutonis PR, Beek TAV (2004) Screening of radical scavenging activity of some medicinal and aromatic plants. Food Chemistry 85:231–237.

Mok MC, Mok DWS, Armstrong DJ, Shudo IO, Isogai Y, Okamoto T (1982) Cytokinin activity of N-phenyl-N0-1,2,3-thidiazol-5- ylurea (thidiazuron). Phytochem 21: 1509-1511.

Monteiro A, Lunn T (1998) Trends and perspectives of vegetable Brassica breeding world-wide, World Conference on Horticultural Research. 17-20 June 1998, Rome, Italy.

Moreno DA, Carvajal M, López-Berenguer C, García-Viguera C (2006) Chemical and biological characterisation of nutraceutical compounds of broccoli. J Pharm Biomed Anal 41: 1508–1522.

Msikita W, Skirvin RM (1989) *In vitro* regeneration from hypocotyl and seedling cotyledons of trochunda (*Brassica oleracea* var. *trochunda* Bailey). Plant Cell Tissue Organ Cult 19: 159-165.

Munshi MK, Roy PK, Kabir MH, Ahmed G (2007) *In vitro* regeneration of cabbage (*Brassica oleracea* var. *capitata*) through hypocotyl and cotyledon culture. Plt Tissue Cultr & Biotech 17: 131- 136.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473-497.

Murata M, Orton TJ (1987) Callus initiation and regeneration capacities of *Brassica* species. Plant Cell Tissue Organ Culture 11: 111-123.

Murthy HN, Hahn EJ, Paek KY (2008) Adventitious roots and secondary metabolism. Chin J Biotechnol 24: 711-716.

Musgrave M (2000) Realizing the potential of rapid-cycling Brassica as a model system for use in plant biology research. J Plant Growth Regul 19: 314-325.

Nagarajan A, Arivalagan U, Rajaguru P (2011) *In vitro* root induction and studies on antibacterial activity of root extract of *Costus igneus* on clinically important human pathogens. J Microbiol Biotech Res 1: 67-76.

Nielsen JK, Olsen CE, Petersen MK (1993) Acylated flavonol glycosides from cabbage leaves. Phytochemistry 34: 539-544.

Niemi K, Julkunen-Tiitto R, Tegelberg R, Haggman H (2005) Light sources with different spectra affects root and mycorrhiza formation in Scots pine in vitro. Tree Physiol 25: 123-128

Nieuwhof N (1969) Cole Crops. London, World Crops Books: 26-56.

Nissen SJ, Sutter EG (1990) Stability of IAA and IBA in nutrient medium of several tissue culture procedures. Hort Sci 25: 800–802

Paek, KY Chakrabarty, D Hahn, EJ (2005). Application of bioreactor system for large scale production of horticultural and medicinal plants. Plant Cell Tiss Org Cult 81: 287-300.

Palmer JD, Shields CR, Cohen DB, Orton TJ (1983) Chloroplast DNA evolution and the origin of amphidiploid *Brassica* species. Theoret Appl Genet 65: 18 l-189.

Parveen S, Shahed A (2011) A micropropagation protocol for *Cassia angustifolia* from root explants. Acta Physiol Plant 33: 789-796.

Patel KG, Patel KV, Shah RR, Yagnik RM, Gandhi TR (2011) Evaluation antiasthmatic potential of spray dried powder of fresh juice of leaves of *Brassica oleracea*e variety *capitata.* Res J of Pharma Biol & Chem Sci 2: 370-378.

Pavlovic S, Vinterhalter B, Mitic N, Adzic S, Pavlovic N, Zdravkovic M, Vinterhalter D (2010) In vitro shoot regeneration from seedling explant in *Brassica* vegetables: Red cabbage, Broccoli, Savoy cabbage and cauliflower. Biol Scie Belgrade 62: 337-345.

Peng - fang ZHU, Li LIU, Guang ZHU (2003) A Study on in vitro culture of *Brassic oleracea* var. *acephala*. cnki:ISSN : 1000- 1700.0.2003- 002.

Pereira DM, Valentao P, Pereira JA, Andrade PB (2009) Phenolics: From Chemistry to Biology. Molecules 14: 2202-2211.

Peters WS, Felle HH (1999) The correlation of profiles of surface pH and elongation growth in maize roots. Plant Physiology 121: 905-912.

Peters WS, Luethen H, Boettger M, Felle H (1998) The temporal correlation of changes in apoplast pH and growth rate in maize coleoptile segments. Australian J of Plt Physiol 25: 21-25.

Philip VJ, Dominic J, Triggs GS, Dickinson NM (1992). Micropropagation of black pepper *(Piper nigrum L.)* through shoot tip cultures. Plt Cell Rep 12: 41-44.

Pierik RLM, Steegmans HHM (2003) Analysis of adventitiousroot formation in isolated stem explants of *Rhododendron.* Sci Horticult 1: 1-20**.**

Plumb GW, Price KR, Rhodes MJ, Williamson G (1997) Antioxidant properties of the major polyphenolic compounds in broccoli. J of Agri & Food Chem 49: 2679-2683.

Rackova L, Oblozinsky M, Kostalova D, Kettmann V, Bezakova L (2007) Free radical scavenging activity and lipoxygenase inhibition of *Mahonia aquifolium* extract and isoquinoline alkaloids. J Inflam 4:15-21.

Rafat A, Aziz MA, Rashid AA, Abdullah SNA, Kamaladini H, Sirchi MHT, Javadi MB (2010). Optimization of *Agrobacterium tumefaciens* mediated transformation and shoot regeneration after co-cultivation of cabbage (*Brassica oleracea* subsp. *capitata*) cv. KY Cross with *At*HSP101 gene. Sci Hort 124: 1-8.

Ramakrishna BS, Varghese R, Jayakumar S, Mathan M, Balasubramanian KA (1997) Circulating antioxidants in ulcerative colitis and their relationship to disease severity and activity. J. Gastroenterol Hepatol 12: 490-494.

Ravanfer SA, Aziz MA, Kadir MA, Haddadi F (2011) *In vitro* adventitious shoot regeneration and acclimatization of *Brassica oleracea* supsp. *Italic* cv. Green Marvel. African J of Biotech 99: 1271-1274.

Ravanfer SA, Aziz MA, Kadir MA, Rashid AA, Sirchi MHT (2009) Plant regeneration of *Brassica oleracea* subsp. *italica* (Broccoli) CV Green Marvel as affected by plant growth regulators. African J of Biotech 8: 2523-2528.

Rayle DL, Cleland RE (1992) The Acid growth theory of auxin-induced cell elongation is alive and well. Plant Physi 99: 1271-1274.

Reinert J, Backs D (1968) Control of totipotency in plant cells growing *in vitro*. Nature 220: 1340-1342.

Rosa EAS (1999) Chemical composition. In: Go´mez-Campo C (ed) Biology of *Brassica* Coenospecies. Elsevier Science B.V, Amsterdam, pp 315–358.

Saifullah A, Hahn EJ, Paek KY (2008) Aeration volume and photosynthetic photon flux affect cell growth and secondary metabolite contents in bioreactor cultures of *Morinda citrifolia*. J Plant Biol 51: 209-212.

Sarıkam G, Balkaya A, Yanmaz R (2009) Glucosinolates within a collection of white head cabbages (*Brassica oleracea* var. *capitata* sub.var. alba) from Turkey. African J of Biotech 8: 5046-5052.

Sas K, Robotka H, Toldi J, Vecsei L (2007) Mitochondrial, metabolic disturbances, oxidative stress and kynurenine system, with focus on neurodegenerative disorders. J Neurol Sci 257: 221-239.

Scalbert A, Johnson IT, Saltmarsh M (2005) Polyphenols: antioxidants and beyond. American J of Clin Nut 81: 215S- 217.

Scott RD, "Chrom-Ed Series; Analyte Categories: Phenols," http://www.chromatographyonline.org/directory/analtcat-50/page.html.Accessed 13October2007

Seelye JF, Maddocks DJ, Burge GK, Mrgan ER (1994) Shoot regeneration from leaf discs of *Limonium perigrinum* using Thidiazuron. New Zealand J Crop & Hort Sci 22: 23-29.

Shadidi F, Nazck M (1995) Food Phenolic Sources Chemistry, Effects Applications. Technomic Publishing Company, Lancaster-Basel 331.

Sheen J (1990) Metabolic repression of transcription in higher plants. Plant Cell 2: 1027-1038.

Sikora E, Cie´slik E, Leszczy´nska T, Filipiak-Florkiewicz A, Pisulewski PM (2008). The antioxidant activity of selected cruciferous vegetables subjected to aquathermal processing. Food Chem. 107: 55–59.

Singh U, Jialal I (2006) Oxidative stress and atherosclerosis. Pathophysiol 13:129-142.

Sivakumar G, Aliboni A, Antonini A, Bacchetta L (2007) Bioactive Sulforaphane from *in vitro* Propagated Brassica Seedlings. Eng Life Sci 7: 275–277.

Smart DR, Kocsis L, Walker AM, Stockert C (2003) Dormant buds and adventitious root formation by Vitis and other woody plants. J Plant Growth Regul 21: 296-314.

Smith MA, Rottkamp CA, Nunomura A, Raina AK, Perry G (2000) Oxidative stress in Alzheimer's disease. Biochim Biophys Acta 1502: 139-144.

Smith MK, Drew RA (1990) Current Applications of Tissue Culture in Plant Propagation and Improvement. Aust J of Plant Physiology 17: 267-289.

Snogerup S (1980). The wild forms of the Brassica oleracea group (2n=18) and their possible relations to the cultivated ones, In: Tsunoda S, Hinata K, Gomez-Campo C (eds)

Brassica crops and wild allies, biology and breeding. Japan scientific press, Tokyo, pp: 121-132.

Soengas P, Sotelo T, Velasco P, Cartea ME (2011) Antioxidant properties of *Brassica* vegetables. Functional Plant Science and Biotechnology 5: 43-55.

Soh W, Cohi E, Cho D (1998) Effects of cytokinin on adventitious root formation in callus cultures of *Vigna unguiculata* (L.) Walp. In vitro Cell Dev Biol Plant 34: 189-195.

Sorin C, Bussell JD, Cammus I, Ljung K, Kowalczky M, Geiss G, Mckhann H, Garcion C, Vaucheret H, Sandberg G, Bellini C (2005) Auxin and Light Control of Adventitious Rooting in Arabidopsis Require ARGONAUTE1. The Plant Cell 17: 1-17.

Sousa C, Lopes G, Pereira DM, Taveira M, Valentao P, Seabra RM, Pereira JA, Baptista P, Ferreres F, Andrade PB (2007) Screening of antioxidant compounds during sprouting of *Brassica oleracea* L. var. *costata* DC. Comb Chem High Throughput Scr 10: 377-386.

Sousa C, Taveira M, Valenta P, Fernandes F, Pereira JA, Estevinho L, Bento A (2008) Inflorescences of *Brassicacea* species as source of bioactive compounds: a comparative study. Food Chem 110: 953-961.

Sousa C, Valentao P, Rangel J, Lopes G, Pereira JA, Ferreres F, Seabra RA (2005) Andrade, P.B. Influence of two fertilization regimens on the amounts of organic acids and phenolic compounds of tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC). J Agri Food Chem 53: 9128-9132.

Spooner D, Hetterscheid W, Van den berg R, Brandenburg W (2003) Plant nomenclature and taxonomy an horticultural and agronomic prospective. Edited by Julis Junick, jphn waleys and sons Inc. pp 27-28.

Szabo MR, Iditoiu C, Chambre D, and Lupea AX (2007) Improved DPPH Determination for Antioxidant Activity Spectrophotometric Assay. Institute of Chemistry, Slovak Academy of Sciences. DOI: 10.2478/s11696-007-0022-7.

Takahashi F, Sato-Nara K, Kobayashi K, Suzuki M, Suzuki H (2003) Sugar-induced adventitious roots in Arabidopsis seedlings. J Plant Res 116: 83-91.

Tanimoto E, Fujii S, Yamamoto R, Inanaga S (2000) Measurement of viscoelastic properties of root cell walls affected by low pH in lateral roots of Pisum sativum L. Plant and Soil 226: 21–28.

The oxford encyclopedia of food and drink in America. New York: Oxford University Press, 2004, 2.

Thiem B, Krawczyk A (2003) Ellagic acid in *in vitro* cultures of *Rubus chamaemorus* L. Herba Pol 49: 202-9.

Tiedink HGM, Davies JAR, Van Broekhoven LW, Vander Kamp HJ, Jongen WMF (1988) Formation of mutagenic N-nitroso compounds in vegetable extracts upon nitrile treatment: a comparison with glucosinolate content. Food and Chemical Toxicology 26: 947-954.

Tracewell CA, Vrettors JS, Bautista JA, Frank HA, Brudvig GW (2001) Carotenoid photooxidation in photosystemΠ. Arch. Biochem Biophys 385: 61-69.

Traka M, Mithen R (2009) Glucosinolates, isothiocyanates and human health. Phytochem. Rev 8: 269-282.

Tripathi L, Tripathi JN (2003) Role of biotechnology in medicinal plants. Tropical J of Pharmacl Res 2: 243-253.

Turgeon C (1977) Of Cabbages and Kings Cookbook – An uncommon collection of recipes featuring that family of vegetables which includes *broccoli*, Brussels sprouts, cauliflower, collards, turnips, kale and kohlrabi. The Curtis Publishing Company.

U N (1935) Genomic analysis in Brassicawith special reference to the experimental formation of *B. napus* and peculiar mode of fertilization. Japan J Bot 7: 389-452.

Ugolini LG, De Nicola, Palmieri S (2008) Use of reverse micelles for the simultaneous extraction of oil, proteins and glucosinolates from cruciferous oilseeds. J Agric Food Chem 56: 1595-1601.

Upston JM, Kritharides L, Stocker R (2003) The role of vitamin E in atherosclerosis. Prog Lipid Res 42: 405-422.

Vallejo F, Tom_as-Barber_an FA, Ferreres F (2004) Characterisation of flavonols in broccoli (Brassica oleracea L. var. italica) by liquid chromatography-UV diode-array detection-electrospray ionisation mass spectrometry. J Chromatogr 1054: 181-193.

Vallejo F, Tomas-Barberan FA Garcia-Viguera C (2002) Potential bioactive compounds in health promotion from broccoli cultivars grown in Spain. J of the Sci of Food and Agri 82: 1293-1297.

Vallejo F, Tomas-Barberan FA, Banavent-Garcia AG Garcia-Viguera C (2003a) Total and individual glucosinolate contents in inflorescences of eight broccoli cultivars grown under various climatic and fertilization conditions. J of the Sci of Food and Agric 83: 307-313.

Vallejo F, Tomas-Barberan, F Garcia-Viguera C (2003b) Health-promoting compounds in broccoli as influenced by refrigerated transport and retail sale period. J Agric Food Chem 51: 3029-3034.

Van Volkenburgh E (1999) Leaf expansion-integrating plant behaviour. Plant Cell & Envi 22: 1463-1473.

Vandemoortele JL, Billiard JP, Boucaud J, Gaspar T (1999). Evidence for an interaction between basal medium and plant growth regulators during adventitious or axillary shoot formation of cauliflower. *In vitro* Cell Dev Biol Plant 35: 13-17.

Vasil IK, Vasil V (1972) Totipotency and embryogenesis in plant cell and tissue cultures. *In vitro* Cell Dev Biol-Plant 8: 117-25.

Velasco P, Francisco M, Moreno DA, Ferreres F, García-Viguera C, Cartea ME (2010) Phytochemical fingerprinting of vegetable *Brassica oleracea* and *Brassica napus* by simultaneous identification of glucosinolates and phenolics. Phytochem Anal 22: 144-152.

Velioglu YS, Mazza G, Gao L, Oomah BD (1998) Antioxidant activity and total phenolics in selected fruits vegetables and grain products. J of Agr & Food Chemi 46: 4113-4117.

Verdeil JL, Alemanno L, Niemenak N, Tranbarger TJ (2007) Pluripotent versus totipotent plant stem cells: dependence versus autonomy? Trends Plant Sci 12:245–52.

Via S, Gomulkiewicz R, De Jong G, Scheiner SM, Schlichting CD, Van Tienderen PH (1995) Adaptive phenotypic plasticity: consensus and controversy. Trends Ecol Evol 19: 212- 217.

Vinson JA, Hao Y, Su X, Zubık L (1998) Phenol antioxidant quantity and quality in foods: Vegetables. J Agric Food Chem 46: 3630-3634

Vita JA (2005) Polyphenols and cardiovascular disease: effects on endothelial and platelet function. American J of Clin Nutr 81: 292 - 297.

Vural H, Es¸iyok D, Duman I (2000) The culture vegetables (vegetable growing). Izmir, Turkey p 440.

Wachsman MB, Lopez EM, Ramirez JA, Galagovsky LR, Coto CE (2000) Anti-viral effect of brassionsteroids against herpes virus and arena viruses. Antiviral Chem & Chemother 11: 71-77.

Wachsman MB, Ramirez JA, Galagovsky LR, Coto CE (2002) Anti-viral activity of brassionsteroids derivatives against measeles virus in cell cultures. Antiviral Chem & Chemother 13: 61-66.

Walter A, Silk WK, Schurr U (2000) Effect of soil pH on growth and cation deposition in the root tip of *Zea mays* L. J of Plt Growth Regu 19: 65-76.

Wang W, Wang S, Howie AF, Beckett GJ, Mithen R, Bao Y (2005) Sulforaphane, erucin, and iberin up-regulate thioredoxin reductase 1 expression in human MCF-7 cells. J Agric Food Chem 53: 1417- 1421.

Warwick SI, Black LD (1993) Molecular relationships in subtribe Brassicinae (Cruciferae, tribe Brassiceae). Can J Bot 71: 906-918.

Warwick SI, Gugel RK, Gómez-Campo C (2007). Genetic variation in the *Eruca vesicaria* (L.) Cav. Plant Genet Resour Charact Util 5: 142-153.

Warwick SI, Sauder C (2005) Phylogeny of tribe Brassiceae based on chloroplast restriction site polymorphisms and nuclear ribosomal internal transcribed spacer (ITS) and chloroplasttrnL intron sequences. Can J Bot 83: 467-483.

Widiyanto SN, Erytrina D (2001). Clonal propagation of broccoli, *Brassica oleracea L.*var. *italica* through *in vitro* shoot multiplication. J Med Sci 6: 101-111.

Withers LA (1980) Preservation of germplasm. In: Vasil IK (Editor), Perspectives in Plant Cell and Tissue Culture, Int. Rev Cytol Suppl 11: 101-136.

Wu CH, Dewir YH, Hahn EJ, Paek KY (2006) Optimization of Culturing Conditions for the Production of Biomass and Phenolics from Adventitious Roots of *Echinacea angustifolia*. J of Plt Biology 49: 193-199.

XiuShu Z, MingYang L, WenLing Z, Fan L (2009) Establishment of high Adventitious shoot regeneration system of Ornamental Kale. Genomics & Applied Biol 28: 141-148.

Yesil-Celiktasa O, Nartopb P, Gurelb A, Bedirb E, Vardar-Sukanb F (2007) Determination of phenolic content and antioxidant activity of extracts obtained from *Rosmarinus officinalis* calli. J of Plt Physiol 164:1536-1542.

Yokota, T (1997) The structure, biosynthesis and function of brassinosteroids. Trends in Plt Sci 2: 137-143.

Zobayed SMA, Saxena PK (2003) In vitro-grown roots: a superior explant for prolific shoot regeneration of St. John's wort (*Hypericum perforatum* L. cv 'New Stem') in a temporary immersion bioreactor. Plant Sci 165: 463-470.

Zobel RW (1986) Rhizogenetics (root genetics) of vegetable crops. Hort Science 21: 956-959.

Zhu XY, Chai SJ, Chen LP, Zhang MF, Yu JQ (2010) Induction and origin of adventitious roots from chimeras of *Brassica juncea* and *Brassica oleracea*. Plt Cell Tiss Organ Cult 101: 287-294.