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Changes of Soluble Sugars and Enzymatic Activities During Adventitious Rooting in Cuttings of *Grewia optiva* as Affected by Age of Donor Plants and Auxin Treatments

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ABSTRACT

Rooting and sprouting traits of single-node leafy cuttings (SNCs) of *Grewia optiva* have been studied in relation to age of donor plants and auxin treatments. SNCs from 1-, 5-, 10- and 15-year-old donors and treated IBA 3000 mg L^{-1} gave the best responses. Periodic sampling was performed to examine Total Soluble Sugar (TTS), Indole Acetic Acid (IAA) oxidase and peroxidase (PER) activities in the rooting zone during Adventitious Root Formation (ARF). TTS decreased with passage of time from 0 to 40 days of analysis. Auxin treatments exhibit higher TSS in all donors. IAA-oxidase and PER reduced with maturation. IAA-oxidase was highest in untreated cuttings while auxin treatments had enhanced PER activity. IAA-oxidase was found to decrease up to 20 days and increased thereafter. PER increased with time from the day of planting upto its 30 days, followed by a remarkable decline at 40, irrespective of age of donor plants or auxin treatments. Both enzyme activities remained higher in SNCs of 1-year-old donors which rooted more profusely as compared other donors. Present study suggests auxin treatments have activated carbohydrate metabolism to release energy for ARF. IAA-oxidase and PER activities seems to involve for triggering, initiation and elongation of root primordia.

Key words: *Grewia optiva*, rooting, aging, enzymes, soluble sugars

INTRODUCTION

Adventitious Root Formation (ARF) in stem cuttings is a crucial step in propagation of woody plants and there is a great variability in the rooting ability of different species. While propagation through seeds leads to genetic variability, clonal multiplication has an advantages in developing true-to-type, disease-free varieties of economically and commercially important plants. Several investigations have been made to improve the rooting efficiency of stem cuttings in selected plant species because genetic, environmental and endogenous factors are responsible for ARF (Haissig *et al*., 1992; Haissig and Davis, 1994; Husen and Pal, 2000, 2003a-c; Husen and Pal, 2001; Smart *et al*., 2003; Bakshi *et al*., 2005; Ludwig-Muller *et al*., 2005; Sorin *et al*., 2005; Saranga and Cameron, 2007; Mateja *et al*., 2007; Ragonezi *et al*., 2010). However, there still exist numerous problem in this technique such as poor rooting rate with increasing age of donor plants (Husen and Pal, 2003b, 2006, Osterc *et al*., 2009; Amri *et al*., 2010; Husen, 2011). Thus, stem cutting propagation which has been widely used in many other plants, must be taken into consideration. With respect to poor rooting rate, exogenous application of auxins has a significant positive effect on ARF (Husen and Pal, 2006; Husen, 2008b).

The process of ARF consist three successive but independent phases (induction, initiation and expression) with different requirements. The induction phases comprises molecular and biochemical events without visible changes. The initiation phase is characterized by cell divisions and root primordia organization. The expression phase consists of intra-stem growth of root-primordia and the emergence of roots. The rooting ability of cuttings has been frequently discussed in relation to soluble and storage carbohydrate content (Haissig, 1986; Rapaka *et al*., 2005; Husen and Pal, 2007b; Husen, 2008a, b) because ARF is a high-energy demanding process. It has been repeatedly been shown that total soluble sugars and starch content are higher in auxins-treated cuttings than in control cuttings (Husen and Pal, 2007b; Husen, 2008b). Moreover auxins play an important role in mobilization of carbohydrates in leaves and upper stem, also increased transport to the rooting zone (Haissig, 1986; Husen and Pal, 2001, 2007b; Husen, 2008b). Auxins increased the availability of sugar at the site of ARF by increasing mobilization of starch (Husen and Pal, 2007b; Husen, 2008a) through increased activity of hydrolyzing enzymes (Husen, 2008b). This still remain a fertile field for continued research. A decrease in the rooting efficiency of *Tectona grandis* leafy-stem cuttings due to aging and maturity of donor plants have been reported (Husen and Pal, 2006). Irrespective of age of donor plants, a decline in total soluble sugars and starch content occurs in the rooting zone of cuttings; higher sugar content in this zone is usually associated with highest rooting response (Husen and Pal, 2007b). Current photosynthesis can substantially contribute and translocate sugar to the base of cuttings and thus play an important role in ARF in certain species (Veierskov, 1988; Pellicer *et al*., 2000; Bakshi and Husen, 2002; Rapaka *et al*., 2005).

The oxidative enzymes are widely distributed in higher plants have a remarkable effect on ARF. Changes in Indole Acetic Acid oxidase (IAA-oxidase) activity and peroxidase (PER) activity pattern have been proposed as biochemical markers for the successive rooting phases (Basak *et al*., 2000; Metaxas *et al*., 2004; Rout, 2006; Husen and Pal, 2007b; Husen, 2008b; Li *et al*., 2009). Furthermore many studies showed that, in the course of ARF, the induction period is characterized by a sharp reduction of PER activity and that the initiation phase display an increase while expression phase shows a gradual reduction in PER activity (Basak *et al*., 2000; Husen and Pal, 2007b; Husen, 2008b). Several researchers have been reported a positive correlation between PER activity and ARF. According to these, it appeared that ARF occurred after the cuttings have reached and passed beyond a peak maximum enzyme activity (Basak *et al*., 2000; Metaxas *et al*., 2004; Husen and Pal, 2007b; Husen, 2008b). In addition, the role of axuins in relation to PER activity in the ARF of various plant species was reported (Rout, 2006; Husen and Pal, 2007b; Husen, 2008b). It has been also suggested that auxins conjugates (Hausman, 1993), PER activity (Chibbar *et al*., 1979; Berthon *et al*., 1989; Garcia-Gomez *et al*., 1995; Husen and Pal, 2007b; Husen, 2008b), IAA-oxidase and amylase activities (Quesada *et al*., 1992; Basak *et al*., 2000; Rout, 2006) are involved in regulation auxin levels.

Grewia optiva Drummond (Tiliaceae) is a highly valued multipurpose moderate size tree species that has been yielding green cattle fodder, furniture, medicine, paper, fibre, dyes and fuel wood. Leaves are highly palatable to cattle, rich in protein and other mineral nutrients (Singh, 1982). Furthermore Husen *et al*. (2004) has been reported that *G. optiva* is photosynthetically more active as compared to *Bauhinia purpurea*, *Meila azedarach*, *Celtis australis* and *Quercus leucotrichophora*. This species has been obliterated from the natural forest and occurs in small patches largely cultivated around homesteads in the outer hills up to 2000 m in the North-West and Central Himalayas (Singh, 1982). The tree is heavily lopped during autumn and following winter at around the same time when the tree is in seeding which results that there is always acute

shortage of good quality seed of this species. Considering these facts, it was thought to be of great interest to undertake a systematic study on the vegetative propagation of *G. optiva* and to undertake a comparative performance of various age groups donor plants softwood cuttings rooting in relation to auxin treatments and accompanying biochemical changes. It was reported that juvenile (only 3-month-old donors) single-node leafy cuttings (SNCs) of *G. optiva* resulted higher rooting response without auxins treatment (Husen *et al*., 2003), however, with maturity SNCs may required auxins for ARF as observed in many other plant species. Swamy *et al*. (2002a, b) observed that 250 mg L^{-1} IBA treatment in monsoon season to juvenile (2-year-old) and mature (15-year-old) hardwood cuttings (not SNCs) had given 80 and 70% rooting, respectively.

To date, no studies have attempted to investigate the effect of individual as well as interactive effects of age of donor plants (1-, 5-, 10- and 15-year-old) and auxins treatments (IBA and NAA) on ARF in SNCs of *G. optiva*; and in addition, there is no information over the changes of total soluble sugars and enzymatic activities in relation to ARF in *G. optiva* softwood cuttings as affected by age of donor plants and auxins treatments, therefore, this experiment was designed.

MATERIALS AND METHODS

Plant materials: Phenotypically superior and healthy plants of *Grewia optiva* Drummond (Tiliaceae) belonging to 1-, 5-, 10- and 15-year-old donors were chosen for this experimentation. For 1-year-old donors, seedlings were obtained from Plant Physiology, Forest Research Institute (FRI), Dehra Dun, Uttrakhand (UK), India. For raising this seedling stocks, the fruits were collected at the maturity from Dhaulas and Shivpuri, Dehra Dun, UK, India. Complete protection was provided regularly against disease and insect attacks by foliar spray with fungicide and insecticide, as and when required. For 5-, 10- and 15-year-old donors, trees were carefully selected and marked in different localities Dhaulas and Shivpuri, Dehra Dun, UK, India. Necessary precautions were taken for the uniformity of age, size and vigour and free from disease, insect, pest and physiological disorder. The ortets (donor plants) were growing in the same environments, i.e., in Dehra Dun. The lower branches of 5-, 10- and 15-year-old donors were pruned in the month of April to promote bud sprouting and new shoot formation while in case of 1-year-old donors, the seedling stocks were severed, so that the stem retain only two basal nodes. The open end of stem was coated with chuapatia paste, comprising mixture of 1 g of copper carbonate and 1g of red lead in 1 l blue copper to avoid infection.

Collection and preparation of cuttings: The new shoots which grew on the pruned branches were harvested wrapped in sphagnum moss in the first week of June, from all donor plants and brought to the same day in mist chamber. These harvested shoots at that time were about 2-month-old. For rooting experiment only basal shoots were used and each shoot was made into single-node leafy cuttings (SNCs). Each nodal shoot cutting retained about 25.0 cm^2 leaf area per cutting. The total length of cutting was about 4.0 cm which comprised 1.0 cm internodal portion above the node and 3.0 cm below it. The SNCs collected from 1-, 5-, 10- and 15-year-old donors were prepared and kept separately. All SNCs were treated with 0.05% (w/v) bavistin for 30 min to avoid any fungal attacks during experimentation.

Auxin treatments and design: A non-auxin control, three Indole-3-Butyric Acid (IBA) concentrations and three α-Naphthalene Acetic Acid (NAA) concentrations were used. Thus, seven treatments were obtained for each age group of donor plants. Auxin treatments were the non-auxin

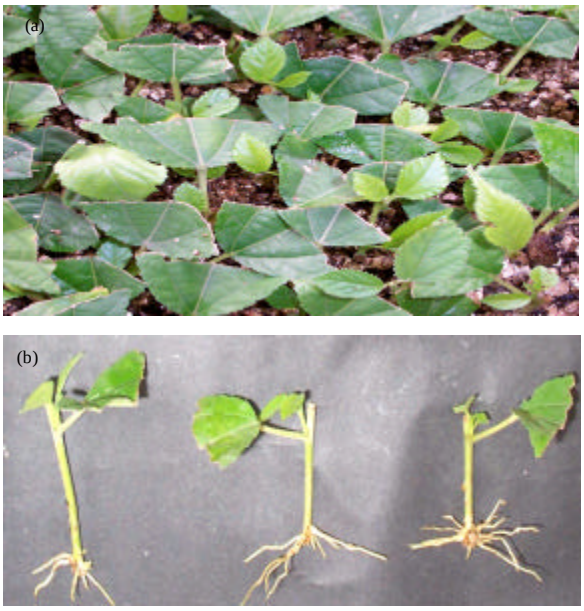

Fig. 1(a-b): (a) Single-node leafy cuttings of *Grewia optiva* inserted in vermiculite and kept inside the mist chamber for rooting and sprouting response and (b) adventitious root formation and shoot growth

control, IBA 1000 mg L^{-1}, IBA 2000 mg L^{-1}, IBA 3000 mg L^{-1}, NAA 1000 mg L^{-1}, NAA 2000 mg L^{-1} and NAA 3000 mg L^{-1}. The auxin solutions were prepared individually by dissolving the appropriate amount of IBA and NAA in 50 mL of 70% alcohol then using de-ionized water to bring the solution to 100 mL. The prepared auxin solutions were stored at 4°C in opaque bottles and used on the same day. The basal end of SNCs obtained from each donor was treated accordingly by quick-dip method. The control SNCs were treated with only alcohol and de-ionized water solution. A randomized complete block design was employed. Five replications were used, with 10 SNCs per treatment per donor plant.

Mist chamber management: After treatments, the SNCs were inserted into sterilized vermiculite (pH 7.0) presoaked in water for 24 h. Then they were cultured in trays inside a mist chamber at 32/26°C (day/night) and 85±2% RH; the automatic day/night misting cycle was set at 60/30 sec, with 1 h delay between successive cycles (Fig. 1a).

Rooting data: Rooting and sprouting on SNCs occurred one week after planting. After 40 days, the cuttings were carefully removed from the rooting medium (Fig. 1b) and evaluated (presence of at least one root/or shoot greater than 0.50 cm in length) for rooting percentage, number of roots per cutting, average root length (cm), sprouting percentage, number of shoots per cutting and average shoot length (cm).

Collection of samples for soluble sugars and enzymatic studies: The rooting zone (~ 0.5 cm) of every age group of donor plants (1-, 5-, 10- and 15-year-old) treated with 1000, 2000 and 3000 mg L^{-1} IBA and NAA along with control SNCs sampled at 0 day (prior to planting in

vermiculite), 10, 20, 30 and 40 day for the estimation of Total Soluble Sugars (TSS), peroxidase (PER) and indole acetic acid oxidase (IAA-oxidase) activities. There were five replications with 10 SNCs per replicate. Each replicate contained five composite samples such that 2 SNCs segments of rooting zone are combined together.

Analysis of total soluble sugar and enzymatic activities: Extract for TSS of SNCs tissues were prepared, according to Sawhney *et al.* (1968) and TSS content was estimated by the phenol-sulfuric acid method (Dubois *et al.*, 1956). PER activity was measured using guaiacol as substrate following Husen (2008b). The assay mixture contained 0.1 M phosphate buffer (pH 6.1), 4 mM guaiacol, 3 mM H_2O_2 and 0.4 mL of crude enzyme extract. The total reaction volume was 1.2 mL. Optical Density (OD) was measured at 420 nm using a Perkin Elmer Lambda 25 UV VIS Spectrophotometer and levels of enzyme activity were expressed as μmol H_2O_2 destroyed min^{-1} mg^{-1} protein. IAA-oxidase activity was determined by the method of Liu *et al.* (1996). Reaction mixture contained 0.2 mL of enzyme extract, 0.78 mL of 50 mM potassium phosphate buffer (pH 6.0), 0.01 mL of 5 mM $MnCl_2$, 0.01 mL of 5 mM 2,4 dichlorophenol, 50 μg of IAA for 30 min at 37°C in dark. The reaction was terminated with 2.0 mL of Salkowski reagent. The destruction of IAA was determined by measuring OD at the reaction mixture at 535 nm after 30 min using a Perkin Elmer Lambda 25 UV VIS Spectrophotometer and the amount of IAA-oxidase activity was expressed as the amount of IAA oxidized μg h^{-1} g^{-1} tissue of rooting zone.

Statistical analysis: The data on the effect of age of donor plants (1-, 5-, 10- and 15-year-old) and auxin treatments (1000, 2000 and 3000 mg L^{-1} IBA and NAA) on the rooting and sprouting response were subjected to two factors analysis, employing analysis of variance (ANOVA). The data obtained for TSS, PER, IAA-oxidase activities were subjected for three factors ANOVA, i.e., age of donor plants, auxin treatments and day of analysis. All the means were compared by using Tukey's test at significance level $p<0.01$ and $p<0.05$. The SPSS/PC software Ver. 16.0 was used to process all the data.

RESULTS

Rooting and sprouting characteristics: After 40 days, the results showed that the SNCs taken from the mature donors significantly decreased rooting and sprouting traits (Table 1). Thus, the highest rooting percentage (71.65%), average root length (5.24 cm) and number of roots (6.74) per SNC was recorded in 1-year-old donors whereas these values were lowest in 15-year-old donor plants (Table 1). Likewise, the highest sprouting percentage (72.35%), average shoot length (1.04 cm) and number of shoots (2.37) per SNC was recorded in 1-year-old donors as compared to the 15-year-old donor plants (Table 1).

Table 1: Effect of age of donor plants on rooting and sprouting of softwood cuttings in *Grewia optiva*

	Growth parameters					
Age of donor plants	(%) rooting	(%) sprouting	Shoots/cutting	Shoot length (cm)	Roots/cutting	Root length (cm)
1 year	71.65^{a}	72.35^{a}	2.37^{a}	1.04^{a}	6.74^{a}	5.24^{a}
5 year	66.33^{b}	65.68^{b}	2.11^{b}	0.99^{b}	5.52^{b}	4.95^{b}
10 year	56.32^{c}	56.04^{c}	1.85^{c}	0.80^{c}	5.12^{c}	4.50^{c}
15 year	53.97^{d}	53.00^{d}	1.62^{cd}	0.71^{cd}	4.69^{d}	3.88^{d}

Values followed by the same letter indicate no significant different at $p<0.05$ level according to Tukey's test

Table 2: Effect of auxin treatments on rooting and sprouting of softwood cuttings in *Grewia optiva*

	Growth parameters					
Auxin treatments (mg L^{-1})	(%) rooting	(%) sprouting	Shoots/cutting	Shoot length (cm)	Roots/cutting	Root length (cm)
Control	55.50^{d}	56.15^{d}	1.22^{d}	0.74^{c}	3.92^{d}	3.71^{d}
IBA 1000	58.62^{c}	61.05^{c}	2.18^{c}	0.94^{b}	4.50^{c}	3.96^{c}
IBA 2000	63.80^{b}	64.87^{b}	2.40^{b}	1.00^{ab}	5.06^{b}	4.53^{b}
IBA 3000	68.69^{a}	71.75^{a}	2.62^{a}	1.04^{a}	7.53^{a}	5.19^{a}
NAA 1000	59.16^{c}	58.96^{c}	1.70^{c}	0.93^{b}	5.17^{b}	4.25^{b}
NAA 2000	63.56^{b}	60.61^{b}	1.91^{bc}	0.82^{c}	6.19^{b}	5.10^{a}
NAA 3000	65.14^{b}	58.98^{c}	1.87^{c}	0.75^{c}	6.27^{a}	5.74^{a}

Values followed by the same letter indicate no significant different at $p<0.05$ level according to Tukey's test

Table 3: ANOVA result on the effect of age of donor plants, auxin treatments and their combination on rooting and sprotuing traits (MSS mean square value * and ** significance level at $p<0.05$ and $p<0.01$, respectively, ns non significant)

	Age of donor plants (A)			Auxin treatments (T)			A×T		
Parameters	MSS	p<0.05	p<0.01	MSS	p<0.05	p<0.01	MSS	p<0.05	p<0.01
(%) rooting	2433.75	-	**	406.77	-	**	58.87	-	**
(%) sprouting	2765.92	-	**	527.59	-	**	41.15	-	**
Shoots/cutting	3.67	-	**	4.33	-	**	0.20	-	**
Shoot length (cm)	0.84	-	**	0.28	-	**	0.01	-	ns
Roots/cutting	27.32	-	**	29.99	-	**	0.74	-	**
Root length (cm)	12.37	-	**	10.74	-	**	0.32	*	-

The SNCs pretreated with auxins were significantly enhanced rooting and sprouting characteristics than those of control cuttings (Table 2). IBA and NAA pretreatments induced different responses. With increasing concentration of IBA 1000 to 3000 mg L^{-1}, there was an increase in rooting efficiency. IBA 3000 mg L^{-1} gave highest percent rooting (68.69%) and number of roots (7.53) per SNC, whereas NAA 3000 mg L^{-1} was best for average root length (5.74 cm). With increasing concentration of IBA, there was an increase in sprouting efficiency. NAA exhibits an increasing trend from 1000 to 2000 mg L^{-1} and again decreasing trend while the dose was increased, i.e., 3000 mg L^{-1} decreased sprouting percentage (58.98%), average shoot length (0.75) and number of shoots (1.87 cm) per SNC. Although, lower concentrations of NAA 1000 and 2000 mg L^{-1} were primitive for these responses (Table 2). For the sprouting traits, IBA 3000 mg L^{-1} gave highest percent sprouting (71.75%), average shoot length (1.04 cm) and number o shoots (2.62) per SNC (Table 2). Except for average shoot length, the interactive effect of age of donor plants and auxin treatments were significant for all rooting and sprouting characteristics (Table 3). The SNCs taken from 1-year-old donors and treated with IBA 3000 mg L^{-1} have shown highest percent rooting (77.93%), percent sprouting (80.35%), average root length (6.28 cm), number of shoots (2.95) and roots (8.23) per SNC. Contrary to this, the control cuttings taken from the 15-year-old donors have given lowest percent rooting (44.33%), percent sprouting (45.38%), average root length (3.14 cm) and number of roots (3.11) per SNC. And, NAA 3000 mg L^{-1} treatment to the 15-year-old donors gave lowest number of shoots (1.07) per SNC (Table 4). In general, the overall best rooting and sprouting responses were occurred by IBA 3000 mg L^{-1} treatment with 1-, 5-, 10- and 15-year-old donors.

Table 4: Interactive effect of age of donor plants and auxin treatments on rooting and sprouting of softwood cuttings in *Grewia optiva*

Growth parameters and age of donor plants	Auxin treatments (mg L^{-1})						
	Control	IBA 1000	IBA 2000	IBA 3000	NAA 1000	NAA 2000	NAA 3000
(%) rooting							
1 year	67.45	70.73	76.90	77.93[a]	71.10	69.93	67.53
5 year	61.95	64.90	67.83	72.31	63.00	67.53	66.76
10 year	48.26	52.26	56.46	60.86	52.46	59.86	64.06
15 year	44.33[b]	46.60	54.00	63.66	50.06	56.93	62.20
(%) sprouting							
1 year	70.72	72.28	78.38	80.35[a]	72.01	67.40	65.33
5 year	60.72	65.30	68.32	75.08	63.39	65.52	61.45
10 year	47.80	54.93	57.10	64.53	51.90	57.13	58.93
15 year	45.38[b]	51.70	55.70	67.04	48.54	52.40	50.23
Shoots/cutting							
1 year	1.33	2.69	2.80	2.95[a]	2.02	2.29	2.49
5 year	1.30	2.18	2.47	2.76	1.59	2.14	2.33
10 year	1.16	2.12	2.24	2.58	1.60	1.66	1.61
15 year	1.09	1.72	2.08	2.20	1.59	1.57	1.07[b]
Shoot length (cm)							
1 year	0.94	1.03	1.15	1.20[a]	1.06	0.96	0.92
5 year	0.88	1.00	1.12	1.13	1.04	0.96	0.83
10 year	0.65	0.85	0.88	0.93	0.87	0.72	0.68
15 year	0.51[b]	0.78	0.86	0.91	0.73	0.64	0.56
Roots/cutting							
1 year	4.73	5.04	6.06	8.23[a]	6.44	7.33	9.37
5 year	4.23	4.71	5.02	6.05	5.11	6.11	7.40
10 year	3.60	4.21	4.79	5.29	4.85	6.12	7.01
15 year	3.11[b]	4.04	4.35	5.51	4.28	5.18	6.35
Root length (cm)							
1 year	4.22	4.43	5.34	6.28[a]	4.68	5.53	6.21
5 year	3.97	4.11	4.89	5.49	4.74	5.31	6.13
10 year	3.51	4.01	4.11	5.01	4.42	4.99	5.44
15 year	3.14[b]	3.29	3.78	3.98	3.17	4.58	5.19

[a, b]Reflects highest and lowest value, respectively

Changes of total soluble sugars and enzymatic activities: The content of Total Soluble Sugars (TSS) in the rooting zone of SNCs varied significantly with age of donor plants, auxin treatments and day of analysis (Table 5). A decrease in TSS was observed with the increase in age of donor plants; i.e. the highest (42.42 mg g^{-1} DW) TSS was recorded in cuttings of 1-year-old donor plants followed by those 5 year (40.77 mg g^{-1} DW), 10 year (39.28 mg g^{-1} DW) and the lowest (38.11 mg g^{-1} DW) in cuttings of 15-year-old donors (Table 6). Exogenous treatments of IBA and NAA increased TSS content in the rooting zone of SNCs. The highest (41.48 mg g^{-1} DW) was recorded by IBA 3000 mg L^{-1} while the lowest (39.04 mg g^{-1}DW) was found in control SNCs. Initially TSS was higher and decreased in SNCs after planting. The highest (39.04 mg g^{-1} DW) TSS was observed at the time of SNCs were planted for rooting; i.e., at the 0 day while the lowest values (21.62 mg g^{-1} DW) estimated at the 40 days of analysis after planting (Table 6). All two factor interactions were significant for TSS (Table 5). The interaction (age of donor plants x auxin treatments) exhibits maximum (45.27) TSS content in SNCs of 1-year-old donor plants, when

Table 5: ANOVA result on the effect of age of donor plants, auxin treatments, day of analysis and their combination on the changes of total soluble sugar, IAA oxidase and peroxidase activities

	Total soluble sugar (mg g^{-1} DW)			IAA-oxidase activity (IAA oxidized $\mu g h^{-1} g^{-1}$ tissue)			Peroxidase activity (µmol H_2O_2 destroyed min^{-1} mg^{-1} protein)		
Source of variation	MSS	p<0.05	p<0.01	MSS	p<0.05	p<0.01	MSS	p<0.05	p<0.01
Age of donor plants (A)	122.04	-	**	166.64	-	**	104.19	-	**
Auxin treatments (T)	14.87	-	**	20.29	-	**	39.69	-	**
Day of analysis (D)	199.58	-	**	444.89	-	**	648.95	-	**
A×T	1.75	*	-	0.51	*	-	1.27	*	-
A×D	5.03	-	**	1.12	-	**	2.74	-	**
T×D	3.34	-	**	1.91	-	**	5.81	-	**
T×A×D	2.11	ns	-	0.87	ns	-	1.88	ns	-

MSS: Mean square value * and ** significance level at p<0.05 and p<0.01, respectively, ns: Non significant

Table 6: Changes of total soluble sugar, IAA oxidase and peroxidase activities during adventitious root regeneration of *Grewia optiva* single-node leafy cuttings (SNCs) as affected by age of donor plants, auxin treatments and day of analysis

Variables	Total soluble sugar (mg g^{-1} DW)	IAA-oxidase activity (IAA oxidized $\mu g h^{-1} g^{-1}$ tissue)	Peroxidase activity (µmol H_2O_2 destroyed min^{-1} mg^{-1} protein)
Age of donor plants			
1 year	42.42^{a}	16.35^{a}	20.57^{a}
5 year	40.77^{a}	15.83^{b}	19.61^{b}
10 year	39.28^{b}	12.46^{c}	17.36^{c}
15 year	38.11^{bc}	12.21^{c}	17.02^{c}
Auxin treatments (mg L^{-1})			
Control	39.04^{d}	15.78^{a}	15.06^{d}
IBA 1000	39.63^{c}	14.15^{d}	16.04^{c}
IBA 2000	40.06^{b}	14.53^{c}	17.38^{b}
IBA 3000	41.48^{a}	15.03^{b}	18.87^{a}
NAA 1000	39.64^{c}	13.90^{e}	15.86^{d}
NAA 2000	40.06^{b}	14.75^{c}	17.26^{b}
NAA 3000	41.10^{b}	14.99^{b}	18.67^{a}
Day of analysis			
0 day	39.04^{a}	18.52^{c}	16.49^{e}
10 day	36.92^{b}	14.16^{d}	17.37^{d}
20 day	33.75^{c}	12.13^{e}	26.41^{b}
30 day	30.18^{d}	20.56^{b}	29.58^{a}
40 day	21.62^{e}	23.75^{a}	20.88^{c}

Values followed by the same letter indicate no significant different at p<0.05 level according to Tukey's test

treated with IBA 3000 mg L^{-1} while minimum (37.16) was recorded in control cuttings of 15-year-old donors (Fig. 2a). The intreaction (age of donor plants x day of analysis) showed maximum (40.23) TSS content in SNCs of 1-year-old donor plants at 0 day of analysis while minimum (18.82) was occurred at 40 days of analysis in cuttings of 15-year-old donors (Fig. 3a). The interaction (auxin treatments x day of analysis) showed maximum (50.27) TSS content at 30 days of analysis, when SNCs were treated with IBA 3000 mg L^{-1} while minimum (40.23) was recorded at 0 day in all auxin treated and control SNCs rooting zone (Fig. 4a). Three factor interactios (age of donor plants x auxin treatments x day of analysis) effects were insignificant (data not shown) (Table 6).

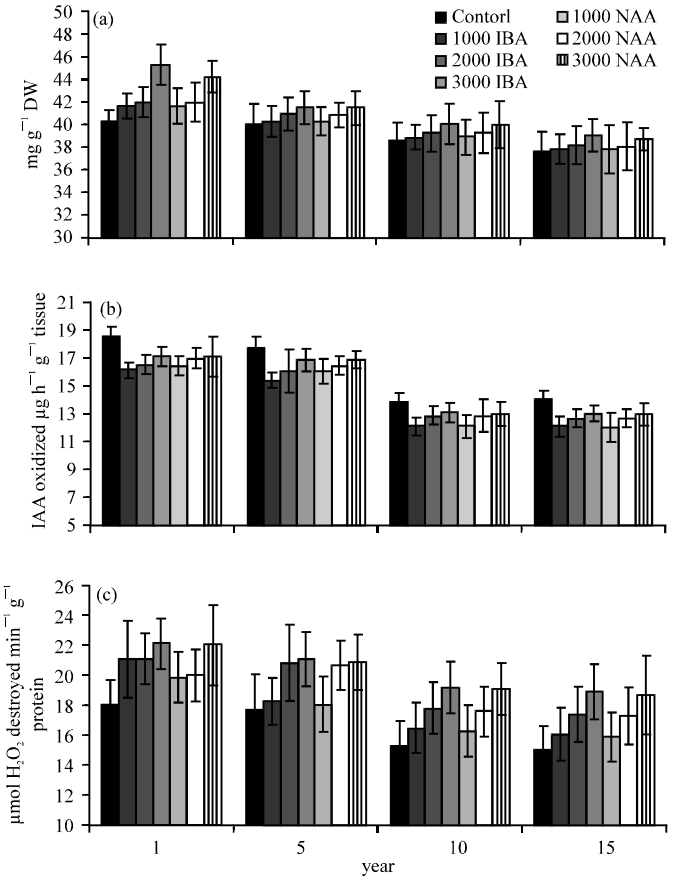

Fig. 2(a-c): Changes in (a) total soluble sugar (b) IAA-oxidase activity and (c) peroxidase activity in rooting zone of *Grewia optiva* cuttings as affected by the interaction of age of donor plants and auxin treatments. Each value represents the Mean±SE of five replicates

The level of IAA-oxidase activity in the rooting zone of SNCs was significantly varied with age of donor plants, auxin treatments and day of analysis (Table 5). IAA-oxidase activity decreased as age of donor plants increased; the highest (16.35 IAA oxidized µg h^{-1} g^{-1} tissue) was found in cuttings of 1 year followed by those for 5 year (15.83 IAA oxidized µg h^{-1} g^{-1} tissue), 10 year and the lowest (12.46 IAA oxidized µg h^{-1} g^{-1} tissue) in 15-year-old donor plants (Table 6). Auxin treatments decreased IAA-oxidase activity as compared to the control SNCs. The control cuttings exhibited highest activity (15.78 IAA oxidized µg h^{-1} g^{-1} tissue) compared to IBA and NAA treatments; while among the auxin treatments, it was lowest (13.90 IAA oxidized µg h^{-1} g^{-1} tissue) with NAA 1000 mg L^{-1} SNCs. The IAA-oxidase activity was decreased from 0 day up to 20 days of analysis. Thereafter, its shows increasing trend up to 40 day of analysis. Therefore, IAA-oxidase activity was found to be lowest (12.13 IAA oxidized µg h^{-1} g^{-1} tissue) at 20 day of analysis and became highest (23.75 IAA oxidized µg h^{-1}g^{-1} tissue) at the last day of analysis, i.e., 40 days (Table 6). All two factor interactions were significant for IAA-oxidase activity (Table 5). The intreaction (age of donor plants x auxin treatments) exhibited maximum (18.56 IAA oxidized µg h^{-1} g^{-1} tissue) IAA-oxidase activity in control SNCs of 1 year old donors while minimum (12.03 IAA oxidized µg h^{-1} g^{-1} tissue) was recorded with NAA 1000 mg L^{-1} treated cuttings of 15-year-old donor plants (Fig. 2b). The intreaction (age of donor plants x day of analysis) showed maximum

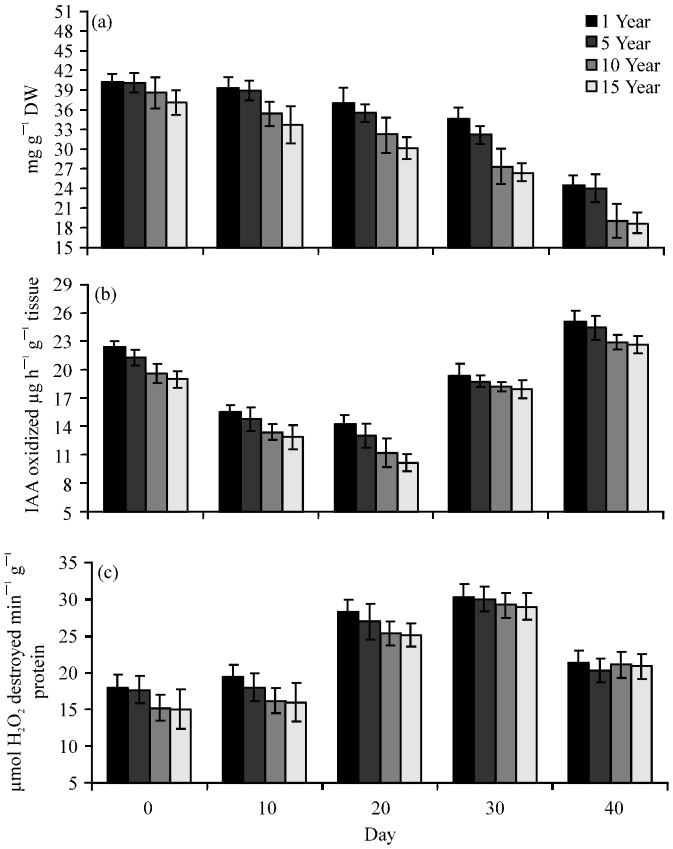

Fig. 3(a-c): Changes in (a) total soluble sugar (b) IAA-oxidase activity and (c) peroxidase activity in rooting zone of *Grewia optiva* cuttings as affected by the interaction of day of analysis and age of donor plants. Each value represents the Mean±SE of five replicates

(25.02 IAA oxidized µg h^{-1} g^{-1} tissue) IAA-oxidase activity in SNCs of 1-year-old donors at 40 days while minimum (10.23 IAA oxidized µg h^{-1} g^{-1} tissue) was found at 20 day of analysis in cuttings obtained from 15 year old donors (Fig. 3b). The interaction (auxin treatments x day of analysis) showed maximum (31.25 IAA oxidized µg h^{-1} g^{-1} tissue) IAA-oxidase activity at 40 day of analysis in control SNCs while minimum (14.52 IAA oxidized µg h^{-1} g^{-1} tissue) was recorded at 20 day with NAA 1000 mg L^{-1} treated cuttings (Fig. 4b). Changes in IAA-oxidase activity due to three factor interactions (age of donor plants x auxin treatments x day of analysis) effects were insignificant (data not shown) (Table 5).

The activity of peroxidase (PER) in the rooting zone of SNCs was significantly varied with age of donor plants, auxin treatments and day of analysis (Table 5). PER activity decreased as age of donor plants increased. The highest (20.57 µmol H_2O_2 destroyed min^{-1} mg^{-1} protein) activity was found in cuttings of 1 year followed by those for 5 year (19.61 µmol H_2O_2 destroyed min^{-1} mg^{-1} protein), 10 year (17.36 µmol H_2O_2 destroyed min^{-1} mg^{-1} protein) and the lowest (17.02 µmol H_2O_2 destroyed min^{-1} mg^{-1} protein) in 15-year-old donor plants (Table 6). Auxin traeatments increased PER activity in the rooting zone as compared to control SNCs. The highest (18.87 µmol H_2O_2 destroyed min^{-1} mg^{-1} protein) activity was recorded by the IBA 3000 mg L^{-1} treatment and it was lowest (15.06 µmol H_2O_2 destroyed min^{-1} mg^{-1} protein) in the control SNCs (Table 6). The lowest (14.49 µmol H_2O_2 destroyed min^{-1} mg^{-1} protein) PER activity was observed at the time of cutting

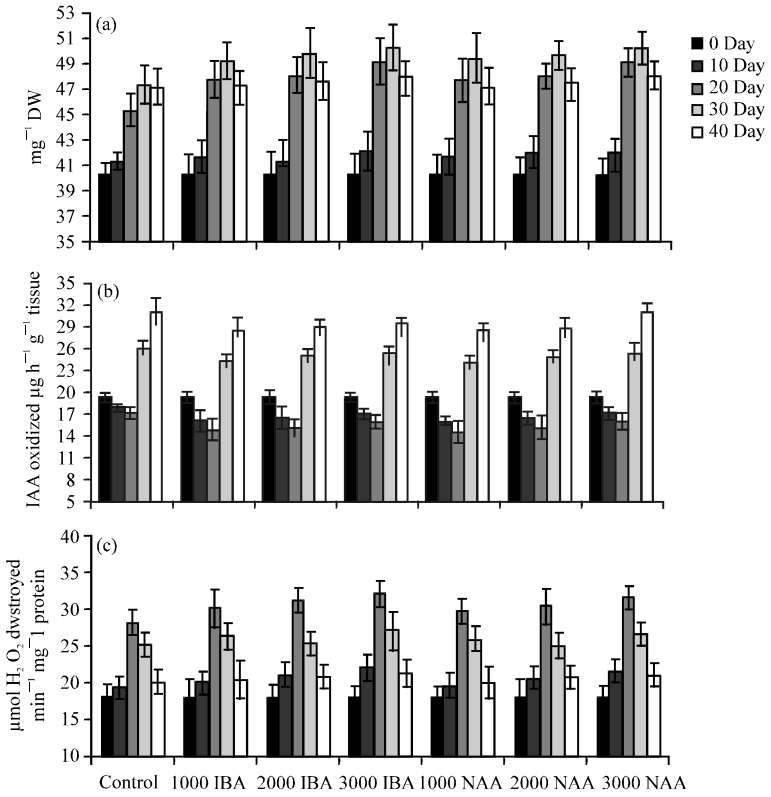

Fig. 4(a-c): Changes in (a) total soluble sugar (b) IAA-oxidase activity and (c) peroxidase activity in rooting zone of *Grewia optiva* cuttings as affected by the interaction of day of analysis and auxin treatments. Each value represents the Mean±SE of five replicates

planting for rooting and it became highest (29.58) at 30 days of analysis. Thereafter, it shows decreasing trend upto the last day of analysis, i.e., 40 days (20.88 µmol H_2O_2 destroyed min^{-1} mg^{-1} protein) (Table 6). For the PER activity, all two factor interactions were significant (Table 4). The interaction (age of donor plants x auxin treatments) exhibited maximum (22.10) PER activity in SNCs of 1-year-old donors, when treated with IBA 3000 mg L^{-1} while minimum (15.06) was recorded in control cuttings of 15-year-old donor plants (Fig. 2c). The intreaction (age of donor plants x day of analysis) showed maximum (30.17) PER activity in SNCs of 1-year-old donors at 30 days while minimum (15.06) was estimated at 0 day of analysis in cuttings obtained from 15-year-old donors (Fig. 3c). The interaction (auxin treatments x day of analysis) showed maximum (32.01) PER activity at 20 day of analysis when SNCs were treated with IBA 3000 mg L^{-1} while minimum (18.03) was recorded at 0 day in all IBA and NAA treated cuttings together with control SNCs (Fig. 4c). Variation in PER activity due to three factor interactions (age of donor plants x auxin treatments x day of analysis) effects were insignificant (data not shown) (Table 5).

DISCUSSION

The present study showed that both rooting and sprouting traits in SNCs declined with increasing age of donor plants. Many other studies with several plant species have shown that ability of ARF decreases with increasing age of donor plants (Husen and Pal, 2006, 2007b;

Osterc *et al*., 2009; Amri *et al*., 2010; Husen, 2011). Previously, SNCs obtained from 3-month-old donors of *G. optiva* gave maximum rooting and sprouting (Husen *et al*., 2003) while in present investigation the minimum age of donor plants was 1-year-old; and therefore, perhaps SNCs required IBA 3000 mg L^{-1} for highest rooting and sprouting. Aging decreased ARF due to a decrease in required endogenous auxins content or root promoters, accumulation of root inhibitors, decreased sensitivity of tissue to auxins with maturity of donor plants and or due to decreased rate of net photosynthesis and carbohydrate metabolism (Hackett and Murray, 1993; Greenwood and Hutchison, 1993; Haissig and Davis, 1994; Bakshi and Husen, 2002; Husen and Pal, 2007b).

Pretreatment of auxins to SNCs improved rooting and sprouting traits of this multipurpose plant. The same results have been reported on many other species capable of rooting (Husen, 2003, 2004, 2008b; Husen and Pal, 2006, 2007c; Amri *et al*., 2010). However, the primitive effect varied with auxin concentrations and types of auxin. IBA 3000 mg L^{-1} produced the best rooting and sprouting traits and hence, it was recomended for *G. optiva*. Higher concentration of NAA did not substaintially produce a better rooting and sprouting results. Moreover, the SNCs pretreated with NAA 3000 mg L^{-1} showed reduction and rooted poor than those pretreated with IBA 3000 mg L^{-1}. The inhibitory effect caused by high NAA treatment also occurred in other plant such as *Tectona grandis* (Husen and Pal, 2006). High auxin treatment can produce toxicity and NAA is more toxic than IBA (Husen *et al*., 2003) in case of 3-month-old donors, therefore, lower NAA and higher IBA concentration were primitive. However, the requirement of auxins concentration may vary with maturity of donor plants due to decrease in the endogenous content of auxin and/or decreased sensitivity of tissues to auxins with physiological aging of the stock (Husen and Pal, 2006). Except average root length, all studied rooting and sprouting traits were found highest due to IBA 3000 mg L^{-1} application in all age groups of donor plants. Therefore, according to current results, if *G. optiva* donor plants reached to the maturity, IBA 3000 mg L^{-1} was recomended to achieve the best rooting and sprouting response.

The results of this study indicated that ARF in SNCs due to age of donor plants, auxin treatments and day of analysis were related to the variation of soluble sugars and enzymatic activities. TSS in rooting zone, irrespective of age of donor plants, decreased with passage of time. For this decrease it has been suggested that stored carbohydrates, namely sugars and starch are mobilized by the activities of hydrolytic enzymes and translocated to the rooting zone of cuttings where they utilized to provide the essential energy for cellular division and differentiation during ARF (Haissig, 1986; Haissig and Davis, 1994; Husen and Pal, 2001, 2007b; Husen, 2008b). Similarly, the results of this study indicated that stored carbohydrate reserves present in SNCs might be utilized during ARF. In addition to this, significant variations in the levels of IAA-oxidase and PER activities were associated with the induction and elongation of root primordial and /or the growth of roots in SNCs. IAA-oxidase activity was higher in 1-year-old SNCs, in comparison to 5-, 10- and 15-year-old donor plants. IBA and NAA treatments have decreased IAA-oxidase activity in SNCs of all age groups while it was remained highest in control cuttings of 1-year-old donors. Further, its activity was decreased in the beginning from 0 day up to 20 days and afterward increased in control as well as auxins treated SNCs while it was remained highest in control cuttings. Therefore, it seems that IAA-oxidase activity in IBA and NAA treated SNCs decreased during induction and initiation phases and increased during expression phase. The results of this study were corroboratory to the other investigators (Nag *et al*., 2001; Rout, 2006). According to Wiesman *et al*. (1988), the low IAA-oxidase activity during the induction period in IBA treated cuttings seems to be responsible for better development of ARF, possibly serving source of free

auxin. PER activity in SNCs, irrespective of age of donor plants increased from the day of their planting for ARF up to the day 30 of analysis and declined subsequently. PER-activity remained higher at all stages of analysis in the SNCs of 1-year-old donors which also rooted more profusely than the SNCs of 5-, 10- and 20-year-old donor plants. An increased PER-activity concomitant with root induction in cuttings has also been reported by others (Haissig, 1986; Haissig and Davis, 1994; Basak *et al*., 2000; Rout, 2006; Husen and Pal, 2007b; Husen, 2008b). The levels of PER activity were also found to be increased due to exogenous IBA and NAA treatments. A similar trend of PER activity was reported in *T. grandis* (Husen and Pal, 2007b) and *Camellia sinensis* (Rout, 2006); and in *Cynara scolymus*, PER activity could be used as a rooting marker in efforts to improve rooting (Moncousin and Gaspar, 1983). Overall studies suggested that both IAA-oxidase and PER activities playing a part in both root initiation and elongation processes (Haissig, 1986; Liu *et al*., 1996; Nag *et al*., 2001; Husen and Pal, 2007a; Husen, 2008b). Treatments of SNCs with IBA or NAA increased TSS in the rooting zone. This increased sugars content during ARF caused by auxin treatments may be attributed to an increase in starch hydrolysis (Haissig, 1984, 1986; Husen and Pal, 2007b; Husen, 2008b) and or increased sugars transport towards rooting zone (Husen and Pal, 2007b). Auxin-carbohydrate relations are also observed to be vital for rooting (Husen and Pal, 2001, 2007b; Husen, 2008b; Haissig, 1986) had reported that the naturally occurring auxin IAA and synthetic auxins affect adventitious rooting of woody stem cuttings. Auxin treatments enhanced initiation of root primordia by stimulating de novo synthesis of specific enzymes, sugars availability through hydrolysis and translocation (Husen and Pal, 2007b; Husen, 2008b). Similarly, the results of this study indicated that both IAA-oxidase and PER activities helps in auxin catabolism and in triggering the root initiation and elongation processes.

CONCLUSION

As a whole, the results presented in this communication suggested that appropriate auxin treatment could produce good rooting response even in case of mature donor plants of *G. optiva*. And, IBA 3000 mg L^{-1} was recommended for 1-, 5-, 10- and 15-year-old donors. In addition, when auxin was applied exogenously, during ARF, TSS increased, IAA-oxidase activity decreased up to 20 days and increased thereafter, PER activity increased up to 20 days and decreased thereafter. These changes during ARF in SNCs suggested that exogenous auxin application activated sugar metabolism for release of energy and enzymatic activities were necessary for cellular division and differentiation.

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