

Some natural extracts from plants as low-cost alternatives for synthetic PGRs in rose micropropagation

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Abstract

Effect of various plant extracts during *in vitro* culture of rose (*Rosa hybrida* L. cv. bush rose), with the objective of replacing synthetic Plant Growth regulators (PGRs) to reduce the production cost, was studied. Test extracts included sweet lime juice, orange juice, sweet corn extract, tomato fruit extract and coconut water. Significant increase in shoot multiplication (15.41 ± 1.12 shoots/explant), shoot length (3.66 ± 0.08 cm), fresh weight (7.48 ± 0.71 g) and dry weight (1.68 ± 0.075 g) was observed when coconut water (@10 % v/v) was used in the standard MS medium. Addition of tomato fruit extract in the MS medium did not show any noteworthy effect on growth in rose micropropagules. Total chlorophyll and other biomolecules varied with the change in the type and concentration of plant extract. Highest accumulation of biomolecules was recorded on coconut water (@ 10 % v/v) supplemented MS medium followed by sweet corn extract and orange juice. Although tomato fruit extract (@10 % v/v) enhanced the total chlorophyll biosynthesis but at the same time depressed the accumulation of other biomolecules. Treatment of plant extract was given in two different ways; a) incorporation in the medium prior to autoclaving (PrA) and b) post-autoclaving addition of filter sterilized extract (PoA). No significant changes were noted in growth when mode of application was changed. To know the physiological pandemonium in the cells, peroxidase and IAA-oxidase activity was noted. No abnormal changes in the activity of these enzymes were recorded in the propagules grown on different plant extracts. The total cost of synthetic 6-benzylaminopurine (BA) can be reduced upto 98 % by replacing it with natural plant extract.

Key words: Rose micropropagation, Synthetic PGRs, natural plant extract, 6-benzylaminopurine, growth, low-cost alternatives

Introduction

Rose is a woody perennial shrub which is native to China but due to its high demand and economic importance, is now grown all over the world. The genus *Rosa* belongs to family Rosaceae which include more than hundred species and enormous commercial cultivars. Multiplication of rose in conventional methods, is generally done by grafting and cutting. But, this method is very slow and risk of disease and environmental hazard is always there (Otiende *et al.*, 2016). To overcome this, a number of tissue culture protocols, which guarantee disease free plantlets, have been developed as a potential tool for rapid and mass propagation in rose (Nikbakht *et al.*, 2005). Micropropagation is not only a gambit for rapid propagation of plants but also overtures elimination of pathogen and endows the researcher with scope for development of new cultivars (Joshi and Purohit, 2011). The last two decades were revolutionary years for commercial tissue culture and the technology in its real sense transformed in profitable business, particularly for ornamental and horticultural plants. Contrary to this, the technology has several limitations. Micropropagation is a cost intensive technology as compared to the conventional methods of plant propagation. It needs several types of skills, accompanied by sophisticated instruments and controlled environmental conditions which make it capital-intensive industry. Hence, in some cases the unit cost per plant becomes exorbitant. This has restricted the growth of these industries in developing countries like India and only a few funded institutions and big companies sustained in this race while small units were dropped out, which has tremendously increased

during past two decade (Singh and Pal, 2015).

There are several factors which add to the production cost of tissue culture grown plantlets. Foremost of these is the cost of chemicals and glassware (which are often very expensive) used in medium preparation. Several studies were carried out for lowering down the production cost of plantlets. These reports were mainly focused on use of some cheaper gelling agents like Xanthagar (Jain-Raina and Babbar, 2011), corn and potato starch (Mohamed *et al.*, 2010), isabgoal (Agrawal *et al.*, 2010), Guar-gum (Babbar *et al.*, 2005), Cotton fiber (Moraes-Cerdeira *et al.*, 1995), use of low grade inorganic salt (Ogero *et al.*, 2012; Dhanalakshmi and Stephan, 2014), use of low grade table sugar as carbon source (Tyagi *et al.*, 2007; Agrawal *et al.*, 2010). The main focus of the researchers was on replacing gelling agent and sucrose as carbon source with some cheaper options, just because both of these components share major cost in medium preparation. Total cost contributed alone by agar-agar (gelling agent) and sucrose (tissue culture grade), in standard MS medium preparation, is 49.61 % and 38.49 % respectively. Beside this, synthetic PGR like 6-BA (7.78 %) and other inorganic and organic component (4.12 %) also significantly contribute to the production cost of standard MS medium which was ignored in this direction. Some natural plant extracts contain important substances same as PGR or which exhibit PGR like activities. The growth promoting activity of coconut water under *in vitro* condition has long back reported as path breaking discovery by Van Overback *et al.* (1941). Beside these important substances, the natural extracts also contains some important amino acids, proteins, carbohydrates, lipids and primary and secondary products which positively influence

the growth of micropropagules under *in vitro* conditions. The artificial MS medium, which are mostly used in plant tissue culture, contain all the essential inorganic and organic salt, chelating agents, carbon source, growth regulators and water. But, the exact nutritional requirements differ from plant to plant and within the same plant differs from cell to cell. The natural plant extract, which are produced through many complex metabolic pathways, contains several metabolites that may act as potent growth enhancers, which a synthetic medium is completely lacking. We hypothesized that the addition of such ready prepared metabolites, in the form of natural extract in the growth medium, can substitute the need of PGRs and thus can lower the production cost as well at the same time promote the growth under culture conditions. In order to increase application of tissue culture technology in rose cultivation, innovative approaches are needed to lower the cost of micropropagules production. The aim of the current study was to develop an efficient and affordable method for the micropropagation of rose.

Materials and methods

Explant preparation and culture establishment: Shoot cultures of *Rosa hybrida* L. cv. bush rose were established according to the protocol described by Carelli and Echeverrigaray (2002). Healthy plants were procured from local nursery and maintained in green house of college. Mature nodal segments, containing axillary buds, were selected, cut and rinsed with tap water to remove the dust particles followed by two washes with detergent and then sterilization by dipping in 70 % ethanol for one min. Further sterilization of explant was done by treatment with 1 % Sodium hypochlorite for 10 min followed by three to four time washing with sterile distilled water. Aseptic inoculation of explant was done on the standard Murashige and Skoog's (1962) medium containing 3.0 mg L⁻¹ BAP, 0.01 mg L⁻¹ NAA, 0.8 % agar and 3.0 % sucrose. After initial establishment of cultures, regular sub-culturing was done every three weeks interval. Cultures were kept under standard growth room conditions maintained at 28±2 °C temperature and a 16 h light/8h dark cycle providing 45µ mol m⁻²s⁻¹ photon flux density.

Experimental design: We tried a range of natural plant extracts viz. orange juice, coconut water, tomato fruit extract, sweet corn extract and sweet lime juice, in varied concentration (3-15 %) to study its regulatory effects on growth of micropropagules. Plant extracts were added to standard multiplication medium of rose devoid of PGR. For comparison, both positive and negative controls were also kept, where the cultures were grown on medium containing PGRs and medium devoid of PGRs, respectively. Since the natural plant extract contain metabolites, which may be thermo-labial and their effect may vary with the autoclaving, two different ways were adopted to incorporate the plant extract. In first approach, plant extracts were incorporated in the nutrient medium prior to autoclaving (pre-autoclaving) while in the second case, filter sterilized plant extracts were added aseptically in the medium after autoclaving (post-autoclaving), when the temperature of medium was brought down to about 50 °C. Each culture bottle contained ca. 50 mL of semi-solid medium and were capped tightly to avoid contamination. The pH of medium was always adjusted to 5.8 before autoclaving. Each culture bottle was aseptically inoculated with a cluster of five shoot (ca. 1.5 cm) and kept under standard growth room conditions as mentioned

earlier. The treated shoots were further sub-cultured on the same plant extract containing fresh medium at a gap of three weeks up to six cycle *i.e.*, a period of 126 days. All the above treatments were repeated thrice and six replicates were set for each experiment. At the end of experiment the micropropagules were taken out of culture bottle and were subjected to measurement of various growth parameters and biochemical analysis.

Measurement of growth parameters: Average shoot length, total shoot number and total biomass in terms of fresh and dry weight were measured. For measurement of biomass (fresh weight and dry weight), propagules obtained from each treatment were taken out and the fresh weight was measured using an electronic top pan balance. For dry weight calculation, after measuring the fresh weight those fresh shoots were kept in an oven at 62 °C for drying till constant weight.

Chlorophyll contents: The chlorophyll contents were calculated as per the method described by Arnon (1949). 500 mg of shoots were weighed and ground in pestle and mortar with 80 % acetone under dark conditions. Extracts were centrifuged at 10, 000 rpm and the supernatant was used to measure absorbance on spectrophotometer (UV-1800 Shimadzu, Japan).

Total phenols: The total phenol content was measured as per the method described by Mahadevan (1975) using Folin Ciocalteu's reagent. 500 mg of shoots were weighed and crushed in pestle and mortar in 70 % methanol. The extracts were centrifuged at 10, 000 rpm for 15 minutes. The clear supernatants were used for quantitative determination of total phenol content. For each reaction 500 µL methanolic extract was taken in a test tube and to this 1.0 mL suitably diluted (1:1 ratio of reagent and DDW) Folin Ciocalteu's reagent was added followed by 2.0 mL of Na₂CO₃ (20 % w/v) solution. The test tubes were heated in boiling water bath with intermittent shaking for about 1.0 min. Tubes were subsequently cooled under running tap water. The blue colored product was diluted to 25 mL by adding DDW and the percent transmittance was measured at 650 nm in a spectrophotometer (UV-1800 Shimadzu, Japan). The total phenol concentration in each sample was estimated with the help of standard curve prepared using different concentrations (10-100 µg) of caffeic acid.

Total carbohydrates: Quantitative estimation of total carbohydrate content was carried out as per method described by Tandon (1976). *In vitro* derived propagules were homogenized in 0.1 M phosphate buffer (pH 7.0) and the homogenates were centrifuged at 10, 000 rpm for 15 min. For each reaction 15 µL of supernatant was mixed with 4.0 mL of 0.2 % Anthrone reagent (in conc. H₂SO₄) and placed in boiling water bath for five minutes. The absorbance was recorded at 610 nm wave length. The total carbohydrate contents were determined using standard curve prepared from various concentrations of glucose.

Total protein: Quantitative estimation of total protein was performed as per Bradford's method (1976). One mL of the suitably diluted crude tissue extract (the supernatant) was mixed with 5.0 mL of Coomassie Brilliant Blue G-250 dye (Bradford reagent) and transmittance of the resultant solution (coloured complex) was read with spectrophotometer (UV-1800 Shimadzu, Japan) at 595 nm. The amount of protein was determined using standard curve prepared using various concentrations of albumin protein.

Total proline: Proline in the plant samples was determined following the method described by Bates *et al.* (1973). Proline was extracted from 1.0 g fresh tissue using 10 mL of aqueous sulphosalicylic acid (3.0 %). The homogenate was centrifuged at 10,000 rpm for 15 min. To 2.0 mL of suitably diluted fresh extract, was added 2.0 mL of glacial acetic acid and 2.0 mL of freshly prepared ninhydrin (1.25 g ninhydrin dissolved in a mixture of 30.0 mL warm glacial acetic acid and 20.0 mL of 6 M O-phosphoric acid and used within 24 h when stored at 4°C. The mixture was heated in boiling water bath for 1 h. The reaction was terminated by transferring the reaction tubes to ice and to it was added 4.0 mL of toluene followed by stirring on a cyclomixer (Remi) for 20-30 sec. After thawing it to room temperature, the toluene layer (upper one, pink in colour) was allowed to separate. The percent transmittance was measured at 520 nm and the proline concentration was determined from standard curve prepared using different concentrations of L-proline.

Peroxidase: Peroxidase was determined by the method given in Worthington Enzyme Manual (Anonymous, 1972). The rate of degradation of hydrogen peroxide (H₂O₂) by the enzyme with O-dianisidine as hydrogen donor was determined spectrophotometrically by measuring the rate of colour development at 460 nm. To a 3.0 mL cuvette, 2.7 mL of 0.1 M phosphate buffer (pH 7.0), 0.1 mL of O-dianisidine (1.0 mg/ml dissolved in 30 % methanol) and 0.1 mL enzyme extract were added. At the end, 0.1 mL of H₂O₂ (0.1 M) was added and mixed quickly. The change in per cent transmittance was recorded at 15 sec intervals for 3.0 min.

IAA-oxidase: The IAA-oxidase activity was determined using 0.5 ml, 1.0 mM dichloroindophenol (DCP), 0.5 mL (1 mM) manganese chloride (MnCl₂), 0.2 mL indole acetic acid (150 µg in 1.0 mL concentration) and made up to 2.0 mL with phosphate buffer and 0.1 mL enzyme extract, using method of Srivastava and Van Huystee (1973). The reaction mixture was incubated at 37 °C in dark. After an hour, 2.0 mL of Salkowski's Reagent (1.7 g FeCl₃·6H₂O dissolved in 12 mL distilled water, in an ice bath, 240 mL of H₂SO₄ added very slowly) was added to terminate the reaction in the tubes. The per cent transmittance of the mixture was measured at 530 nm after 1 h incubation. The amount of IAA oxidized was calculated with the help of standard curve of IAA. The activity of IAA oxidase is represented as amount of IAA oxidized by each gram fresh weight of tissues in one hour.

For all the above analysis, three replicates were used and each reaction was repeated thrice. Suitable blanks were maintained wherever required. A statistical analysis was done to check the validity of data.

Result and Discussion

Plant tissue culture is key sector of agri-biotechnology industry in India and floriculture lead the market, supplying mostly the plantlets in the international market of 150 countries all over the world (Malhotra, 2012). Till the end of April 2016, Department of Biotechnology (DBT), Government of India under the "National Certification System for Tissue Culture Raised Plants (NCS-TCP), has recognized 96 plant tissue culture industries, which was initially only one in the year 1985 (http://dbtncstcp.nic.in/html/content/Recognized_TCPUs.pdf. Accessed

June 1, 2016). Major tissue culture raised ornamental plants, produced by these industries for international market are Ficus, Rose, Spathiphyllums, Syngoniums, Philodendrons, Nerium, Alpenia, Yucca, Cordylines, Pulcherrima, Sansevieria, Gerbera, *Anthuriums*, Statis, Lilies, Alstromeria *etc.* The pace of growth of these industries has fallen in recent past, as evident by report from Department of Agriculture and Processed Food Products Export Development Authority, Government of India, which says that export in floriculture was 27121.86 metric ton in the year 2012-13, which was dropped to 22947.27 metric ton in the year 2014-15 (<http://agriexchange.apeda.gov.in/indexp/exportstatement.aspx>. Accessed June 1, 2016). The expansion of these industries is mainly hampered by high production cost of plantlets.

There are several reports where trials for lowering the production cost of plantlets has been done but these were mainly focused on replacing the gelling agent and carbon source, while the PGRs also contribute significant cost in production. In the present study, we tried some natural plant extract *viz.* orange juice, coconut water, tomato extract, sweet corn extract and sweet lime juice as alternative source of costlier synthetic PGRs. In order to study the effect of above mentioned plant extract, several growth parameters *viz.* number of shoot, shoot length, fresh weight and dry weight were recorded and compared with control micropropagules (both grown on with or without standard PGRs). The growth of micropropagules varied when type, concentration and mode of application of plant extracts were changed. Number of shoot were found to be highest (15.41±1.12), when coconut water was added at 10 % (v/v) concentration while sweet lime juice showed marginal increase in shoot number among the other extracts tested (Table 1). Addition of plant extracts after the autoclaving of medium (post-autoclaving) always showed increased number of shoot at the corresponding concentration in all the cases. This may be due to the obvious reason because since high temperature degrades certain thermo labial compounds present in the extract. Among the concentrations of various extracts tested, 10 % (v/v) concentration was found to be best in terms of increase in shoot number except for sweet lime juice where 15 % (v/v) concentration showed better results. Higher concentration (15 % v/v) always hampered the growth in all the cases and this may be due to the fact that with the increased concentrations, number of inhibitory molecules in the extracts also increases which subsequently interfere in growth (Arteca, 2013). Besides the growth regulators, the addition of various plant extract also increase mineral concentration in the medium which indirectly support in increase of shoot number (Niedz *et al.*, 2015). The shoot numbers in treated plants were comparable with the numbers obtained in control plants, where standard PGRs were used (Table 1). Similarly, significant increase in shoot length was also recorded with the addition of different plant extracts. Highest increase in shoot length was observed with sweet corn extract (4.39 cm @ 15 %) which was higher than its corresponding control plant (4.1 cm; Table 1). Both orange juice and sweet lime juice exerted similar effect with respect to shoot length which was comparatively lower than coconut water. Although supplementation of coconut water significantly increased the shoot length but it did not equate with control plants, supplemented with standard PGR. In case of fresh and dry weight, coconut water gave better results followed by sweet corn and orange juice. Coconut water at 10 % (v/v) concentration

Table 1. Effect of different plant extract on *in vitro* growth of rose micropropagules

Plant Extract	Plant extract concentration (%)	No. of shoots (Mean) \pm SD		Length of shoots (cm) \pm SD		Fresh weight (g) \pm SD		Dry weight (g) \pm SD	
		PrA	PoA	PrA	PoA	PrA	PoA	PrA	PoA
Orange juice	3	8.12 \pm 0.73	8.98 \pm 1.11	1.81 \pm 0.04	1.89 \pm 0.04	4.18 \pm 0.10	4.62 \pm 0.06	0.95 \pm 0.031	1.04 \pm 0.036
	5	9.25 \pm 0.86	10.22 \pm 1.17	1.92 \pm 0.06	2.11 \pm 0.05	4.78 \pm 0.20	5.28 \pm 0.04	1.07 \pm 0.038	1.18 \pm 0.039
	10	12.36 \pm 1.23	13.15 \pm 1.16	2.98 \pm 0.05	3.12 \pm 0.06	6.37 \pm 0.42	6.78 \pm 0.48	1.44 \pm 0.056	1.51 \pm 0.059
	15	10.89 \pm 1.03	11.95 \pm 1.14	3.12 \pm 0.11	3.55 \pm 0.09	5.63 \pm 0.32	6.20 \pm 0.42	1.27 \pm 0.046	1.40 \pm 0.051
Coconut water	3	9.14 \pm 1.23	9.05 \pm 0.76	1.85 \pm 0.03	2.10 \pm 0.04	4.72 \pm 0.19	4.65 \pm 0.07	1.06 \pm 0.036	1.04 \pm 0.036
	5	12.14 \pm 1.16	13.21 \pm 1.02	3.11 \pm 0.09	3.55 \pm 0.07	6.29 \pm 0.56	6.82 \pm 0.51	1.41 \pm 0.048	1.41 \pm 0.051
	10	14.45 \pm 1.22	15.41 \pm 1.12	3.22 \pm 0.10	3.66 \pm 0.08	7.48 \pm 0.71	7.44 \pm 0.74	1.68 \pm 0.075	1.67 \pm 0.066
	15	14.26 \pm 1.16	14.82 \pm 1.19	3.16 \pm 0.09	3.56 \pm 0.04	7.35 \pm 0.69	7.28 \pm 0.72	1.65 \pm 0.052	1.63 \pm 0.055
Tomato extract	3	7.89 \pm 0.66	7.23 \pm 0.28	1.84 \pm 0.04	1.92 \pm 0.04	4.08 \pm 0.12	3.73 \pm 0.08	0.92 \pm 0.031	0.84 \pm 0.026
	5	8.55 \pm 1.16	9.82 \pm 0.79	2.21 \pm 0.06	2.32 \pm 0.05	4.43 \pm 0.16	5.08 \pm 0.20	1.02 \pm 0.032	1.15 \pm 0.031
	10	7.36 \pm 0.49	8.73 \pm 0.26	2.36 \pm 0.07	2.51 \pm 0.05	3.79 \pm 0.09	4.05 \pm 0.12	0.83 \pm 0.025	0.91 \pm 0.033
	15	7.78 \pm 0.87	8.21 \pm 0.21	2.19 \pm 0.04	2.41 \pm 0.04	3.98 \pm 0.14	3.72 \pm 0.11	0.89 \pm 0.056	0.83 \pm 0.027
Sweet corn extract	3	8.24 \pm 0.19	9.66 \pm 0.77	3.08 \pm 0.09	3.66 \pm 0.10	4.25 \pm 0.19	4.98 \pm 0.29	0.95 \pm 0.029	1.12 \pm 0.044
	5	10.42 \pm 0.96	11.74 \pm 1.09	3.42 \pm 0.09	3.82 \pm 0.12	5.38 \pm 0.31	6.22 \pm 0.42	1.21 \pm 0.038	1.39 \pm 0.057
	10	12.27 \pm 1.03	13.98 \pm 1.06	4.21 \pm 0.13	4.36 \pm 0.14	6.33 \pm 0.41	6.72 \pm 0.54	1.42 \pm 0.051	1.51 \pm 0.059
	15	11.19 \pm 1.18	13.04 \pm 1.20	4.35 \pm 0.14	4.39 \pm 0.15	5.78 \pm 0.45	6.76 \pm 0.66	1.30 \pm 0.044	1.52 \pm 0.062
Sweet lime juice	3	6.28 \pm 0.06	7.10 \pm 0.11	1.83 \pm 0.04	1.95 \pm 0.04	3.24 \pm 0.08	3.66 \pm 0.12	0.73 \pm 0.019	0.82 \pm 0.030
	5	6.36 \pm 0.13	7.23 \pm 0.19	1.91 \pm 0.06	1.98 \pm 0.04	3.29 \pm 0.10	3.78 \pm 0.21	0.75 \pm 0.020	0.85 \pm 0.034
	10	8.49 \pm 0.93	7.92 \pm 0.16	2.06 \pm 0.06	2.25 \pm 0.06	4.41 \pm 0.17	4.11 \pm 0.23	0.98 \pm 0.056	0.91 \pm 0.042
	15	9.47 \pm 0.10	9.60 \pm 0.10	2.02 \pm 0.06	2.27 \pm 0.05	3.33 \pm 0.11	3.40 \pm 0.14	0.75 \pm 0.021	0.74 \pm 0.034
(+) ve control (with PGRs)		16.06 \pm 2.11	16.17 \pm 3.32	4.10 \pm 0.12	4.15 \pm 0.05	9.32 \pm 0.95	9.35 \pm 0.86	2.10 \pm 0.096	2.08 \pm 0.086
(-) ve control (without PGRs)		6.12 \pm 0.38	6.32 \pm 0.48	1.79 \pm 0.04	1.81 \pm 0.07	3.14 \pm 0.08	3.18 \pm 0.10	0.71 \pm 0.018	0.72 \pm 0.021
LSD ($P=0.05$)		12.12	12.22	2.32	2.30	3.85	3.78	0.0786	0.7617
LSD ($P=0.01$)		14.24	14.32	3.66	3.62	5.51	5.75	0.0953	0.0951
CV		30.21	30.19	32.18	30.19	42.26	40.22	6.51	6.32

SE Standard Error; CD Critical Difference; CV Coefficient of variation SD Standard Deviation; PrA and PoA represent the MS medium where plant extract were added prior to autoclaving and post-autoclaving through sterile filters, respectively.

resulted in increase of fresh weight of propagules upto 7.48 g, which was comparable with that of control plant (Table 1). No morphological abnormalities, callusing or rooting were observed in treated plant at this multiplication stage.

Incorporation of different plant extract at different concentrations evoked varied responses in rose propagules, in terms of biochemical parameters. It was interesting to note that no remarkable changes were observed in terms of biomolecules or enzyme activities, when the mode of application of plant extract was changed. Although addition of these extracts, in sterile condition, after the autoclaving of the medium (PoA), resulted into slight increase of these biomolecules in most of the cases (except the proline), but it did not make any significance. In all the test extracts, except sweet lime juice, the chlorophyll contents increased with the increase in extract concentration and became equivalent to the control plants. The highest chlorophyll content was recorded in the propagules grown on medium containing 10 % (v/v) tomato fruit extract. In contrast, post autoclaving addition of plant extracts did not show any significant increase in total chlorophyll contents (Table 2). Steady increase in total carbohydrate and phenols contents was observed in the rose propagules when grown on medium supplemented with lower concentrations (5 % v/v and 10 % v/v) of all the tested plant extracts. Further increase in concentrations of plant extracts caused decline in amount of both total phenols and carbohydrates but were still higher than their corresponding controls (Table 3). Although significant increase in carbohydrate contents was recorded at 15 % (v/v) concentration of sweet corn extract, but at 5 % (v/v) concentration, addition of coconut water proved better compared to all of the test samples and even the control

plants. In contrary to total carbohydrates, incorporation of even low concentration of all the test extracts (*i.e.*, 3 % v/v), resulted in higher accumulation of phenol content as compared to the controls. This could be explained on the basis of presence of externally supplied glucose (present in plant extracts) medium, which induce the accumulation of phenols in tissue culture propagules (Ozyigit *et al.*, 2007). Similar observation was recorded in scarlet rose by Amorim *et al.* (1977) where exogenous supply of glucose in culture medium resulted in increased accumulation of phenols. Another important reason of increased phenol accumulation is due to increased proline biosynthesis. It is clearly observed in Table 3 that accumulation of phenol and proline are correlated and directly proportionate. Our results are in accordance with observation of Shetty (2004) that proline-linked pentose phosphate pathway stimulate shikimate and phenylpropanoid pathways and this leads to the stimulation of phenol biosynthesis in cell. There are two views on role of phenols under *in vitro* growth and development of plants. One opinion points that the phenols depress *in vitro* proliferation and growth of plants while others talk about the opposite (López *et al.*, 2001). Role of phenols in controlling interaction between PGRs and averting the abscisic acid promoted cell senescence under *in vitro* conditions has also been reported (Feucht and Treutter 1996) which are in agreement of our observation and hypothesis that increase in phenol is not hampering growth of propagules.

Proline is another important metabolite that accumulates under various stress conditions (Qin *et al.*, 2011). Addition of natural extract resulted into increased accumulation of proline. With the increasing concentration of all the test extracts, proline accumulation also increased (Table 3). Highest increase in proline

Table 2. Effect of different plant extract on chlorophyll contents in rose micropropagules grown under *in vitro* conditions

Plant Extract	Plant extract concentration (%)	Total chlorophyll content (mg g ⁻¹ FW ±SD)		Chlorophyll a content (mg g ⁻¹ FW ±SD)		Chlorophyll b content (mg g ⁻¹ FW ±SD)	
		PrA	PoA	PrA	PoA	PrA	PoA
Orange juice	3	0.21 ±0.0041	0.26 ±0.0039	0.11 ±0.0021	0.14 ±0.0035	0.12 ±0.0022	0.12 ±0.0162
	5	0.29 ±0.0203	0.25 ±0.0201	0.18 ±0.0125	0.13 ±0.0086	0.16 ±0.0100	0.11 ±0.0094
	10	0.35 ±0.0223	0.29 ±0.0241	0.19 ±0.0020	0.16 ±0.0037	0.16 ±0.0092	0.14 ±0.0063
	15	0.38 ±0.0234	0.31 ±0.0212	0.23 ±0.0095	0.16 ±0.0102	0.17 ±0.0111	0.15 ±0.0041
Coconut water	3	0.18 ±0.0042	0.19 ±0.0048	0.10 ±0.0020	0.09 ±0.0122	0.09 ±0.0016	0.09 ±0.0049
	5	0.22 ±0.0301	0.18 ±0.0221	0.11 ±0.0121	0.10 ±0.0133	0.08 ±0.0084	0.07 ±0.0079
	10	0.26 ±0.0253	0.20 ±0.0251	0.12 ±0.0021	0.10 ±0.0111	0.11 ±0.0066	0.09 ±0.0098
	15	0.25 ±0.0234	0.19 ±0.0222	0.12 ±0.0096	0.09 ±0.0166	0.10 ±0.0981	0.09 ±0.0116
Tomato extract	3	0.22 ±0.0041	0.19 ±0.0039	0.09 ±0.0020	0.10 ±0.0021	0.10 ±0.0033	0.09 ±0.0019
	5	0.30 ±0.0204	0.25 ±0.0201	0.17 ±0.0115	0.13 ±0.0125	0.15 ±0.0109	0.11 ±0.0097
	10	0.39 ±0.0233	0.36 ±0.0240	0.22 ±0.0119	0.11 ±0.0020	0.16 ±0.0087	0.12 ±0.0088
	15	0.38 ±0.0271	0.31 ±0.0289	0.23 ±0.0211	0.14 ±0.0095	0.19 ±0.0121	0.15 ±0.0041
Sweet corn extract	3	0.18 ±0.0038	0.19 ±0.0046	0.10 ±0.0019	0.10 ±0.0028	0.12 ±0.0022	0.08 ±0.0072
	5	0.26 ±0.0199	0.21 ±0.0220	0.14 ±0.0125	0.11 ±0.0086	0.16 ±0.0100	0.09 ±0.0140
	10	0.32 ±0.0211	0.30 ±0.0235	0.16 ±0.0031	0.11 ±0.0038	0.16 ±0.0092	0.08 ±0.0032
	15	0.32 ±0.0262	0.32 ±0.0310	0.16 ±0.0005	0.12 ±0.0114	0.17 ±0.0111	0.10 ±0.0071
Sweet lime juice	3	0.23 ±0.0032	0.23 ±0.0037	0.12 ±0.0036	0.10 ±0.0027	0.11 ±0.0044	0.12 ±0.0020
	5	0.21 ±0.0192	0.19 ±0.0222	0.11 ±0.0110	0.09 ±0.0093	0.09 ±0.0080	0.09 ±0.0013
	10	0.23 ±0.0214	0.18 ±0.0248	0.12 ±0.0017	0.10 ±0.0104	0.10 ±0.0072	0.08 ±0.0012
	15	0.22 ±0.0301	0.17 ±0.0210	0.12 ±0.0089	0.09 ±0.0113	0.10 ±0.0021	0.08 ±0.0028
(+) ve control	(with PGRs)	0.28 ±0.0053	0.29 ±0.0053	0.15 ±0.0046	0.15 ±0.0092	0.13 ±0.0096	0.14 ±0.0014
(-) ve control	(without PGRs)	0.20 ±0.0062	0.21 ±0.0062	0.11 ±0.0051	0.11 ±0.0069	0.10 ±0.0083	0.10 ±0.0078
LSD (P=0.05)		0.0181	0.0211	0.0118	0.0179	0.0145	0.0156
LSD (P=0.01)		0.059	0.062	0.038	0.082	0.0182	0.0179
CV		4.46	4.68	4.02	4.11	4.22	4.37

SE Standard Error; CD Critical Difference; CV Coefficient of variation SD Standard Deviation; PrA and PoA represent the MS medium where plant extract were added prior to autoclaving and post-autoclaving through sterile filters, respectively.

Table 3. Effect of different plant extract on total carbohydrates, protein, phenol and proline contents in rose micropropagules grown under *in vitro* conditions

Plant Extract	Plant extract concentration (%)	Total Carbohydrate content (mg g ⁻¹ Fresh tissue)±SD		Total Protein content (mg g ⁻¹ Fresh tissue) ±SD		Total Phenol content (mg g ⁻¹ Fresh tissue)±SD		Total Proline content (mg g ⁻¹ Fresh tissue) ±SD	
		PrA	PoA	PrA	PoA	PrA	PoA	PrA	PoA
Orange juice	3	82.21±2.32	84.45±2.92	68.31±3.66	72.27±1.86	1.41 ±0.07	1.42 ±0.06	18.62 ±0.97	17.32 ±1.16
	5	92.26±3.27	96.85±4.12	79.82±4.56	82.53±2.41	1.87 ±0.21	1.45 ±0.01	25.78 ±1.24	20.45 ±1.11
	10	105.85±3.88	110.22±4.68	75.61±3.64	82.42±2.36	2.19 ±0.08	1.69 ±0.04	34.29 ±1.03	31.69 ±2.04
	15	100.78±3.63	112.45±4.20	69.75±5.82	73.21±3.12	2.12 ±0.10	1.52 ±0.02	42.23 ±2.10	33.52 ±2.03
Coconut water	3	84.31±3.12	92.55±2.88	78.22±2.12	82.36±2.32	1.09 ±0.06	0.89 ±0.07	12.68 ±0.91	09.42 ±1.28
	5	107.26±4.16	108.77±3.22	89.78±3.44	96.42±3.36	1.17 ±0.22	0.96 ±0.01	15.86 ±1.42	12.48 ±1.75
	10	107.95±3.78	116.24±4.88	95.54±3.76	99.39±3.63	1.19 ±0.09	1.01 ±0.02	24.31 ±1.14	20.96 ±2.45
	15	111.73±3.83	116.41±5.21	109.28±4.52	109.32±4.09	2.08 ±0.11	1.81 ±0.07	33.18 ±2.20	23.27 ±2.14
Tomato extract	3	78.41±2.82	82.49±3.02	57.41±1.86	62.72±1.74	1.19 ±0.06	1.69 ±0.11	16.26 ±1.90	15.63 ±1.07
	5	81.23±3.69	85.89±4.18	62.72±2.06	65.68±2.40	1.27 ±0.27	1.96 ±0.04	24.55 ±1.29	22.47 ±1.02
	10	96.81±4.47	100.62±4.31	70.52±3.04	72.53±2.31	1.31 ±0.18	1.91 ±0.06	30.33 ±2.02	25.28 ±2.13
	15	92.76±4.33	104.47±4.10	69.64±3.13	73.33±2.11	1.96 ±0.11	2.01 ±0.10	32.18 ±2.85	27.49 ±2.64
Sweet corn extract	3	86.41±3.22	94.58±2.77	74.23±3.11	78.61±2.38	1.08 ±0.07	0.92 ±0.06	13.58 ±0.98	08.90 ±0.79
	5	96.23±4.13	98.66±3.12	86.82±3.84	90.20±4.06	1.15 ±0.32	0.98 ±0.04	18.81 ±1.22	13.33 ±1.76
	10	100.45±3.48	101.26±4.87	96.41±3.93	99.11±3.92	1.29 ±0.07	1.10 ±0.09	20.33 ±1.10	16.36 ±2.05
	15	116.79±4.89	119.51±4.23	90.12±3.52	92.21±3.08	2.12 ±0.10	2.01 ±0.17	20.19 ±1.22	14.52 ±2.54
Sweet lime juice	3	77.42±1.82	80.26±1.92	60.40±1.81	66.64±2.16	1.51 ±0.11	1.62 ±0.13	27.63 ±2.07	25.23 ±2.26
	5	80.32±2.37	84.58±2.42	72.29±2.17	78.21±2.93	2.17 ±0.22	2.45 ±0.17	35.71 ±2.41	33.54 ±2.11
	10	95.78±3.18	102.12±4.13	76.52±2.37	82.54±3.36	2.78 ±0.12	3.59 ±0.17	44.91 ±3.11	41.28 ±3.21
	15	88.97±3.14	96.54±3.11	68.66±2.04	71.84±2.12	2.42 ±0.11	2.55 ±0.09	48.35 ±3.21	45.19 ±3.43
(+) ve control	(with PGRs)	106.78±4.78	104.54±3.48	90.88±4.01	91.12±3.21	0.89 ±0.06	0.87 ±0.04	14.78 ±1.91	14.60 ±1.08
(-) ve control	(without PGRs)	75.96±2.16	77.25±3.77	52.93±2.61	50.71±3.42	0.90 ±0.07	0.89 ±0.08	22.42 ±1.97	21.61 ±1.97
LSD (P=0.05)		21.2436	21.3436	20.4541	21.6562	0.189	0.193	4.4770	4.5781
LSD (P=0.01)		33.3412	33.1241	30.2511	30.5517	0.324	0.339	6.2418	6.1281
CV		17.82	18.02	16.21	16.35	09.26	08.95	4.04	4.10

SE Standard Error; CD Critical Difference; CV Coefficient of variation SD Standard Deviation; PrA and PoA represent the MS medium where plant extracts were added prior to autoclaving and post-autoclaving through sterile filters, respectively.

was noticed in sweet lime extract which was almost double ($27.63 \pm 2.07 \text{ mg g}^{-1}$ fresh tissue) compared to control plants, even at the lowest concentration tested. Interestingly, the coconut water at low concentration, did not show any significant increase in proline. The probable reason behind higher accumulation of proline is due to increased salt level, acidic compounds, phenols and other metabolites present in test extracts and due to adaptive response of plants in culture conditions. Similar observation was recorded by Woodward and Bennett (2005) in *in vitro* cultures of *Eucalyptus camaldulensis* where increased salt concentration resulted in increased proline accumulation.

Studies on the carbohydrate contents shed light on the sugar metabolism and its uptake in plants. As a general observation in all the test samples, increase in extract concentration resulted in enhancement of carbohydrate level although it failed to equate the control plants except in coconut water. Further addition of extract resulted in decrease of carbohydrates accumulation. Coconut water at 5% and 10% (v/v) concentration exerted similar effect on carbohydrate biosynthesis which was comparable to control plant. Similar observations were also recorded for protein accumulation. Coconut water exerted similar effect in terms of protein accumulation when compared to PGR supplemented control plant. Since in the crude extracts of plants, the mixture of growth substance, inhibitors, phenolics and several other metabolites are found in varied concentration. Hence these extracts could not equate the effect in the biochemical changes during plant development which a purified synthetic growth hormone can do (Table 3).

Peroxidase is known to play a role in growth and differentiation and its high activity could be correlated to the process of differentiation that occurred during shoot induction (Thakar and Bhargava, 1999). No significant change was recorded with change in type of plant extract (Fig. 1). Highest activity in all test

sample was recorded at lowest concentration of extract which subsequently decreased with increasing concentration of extract. The difference in higher and lower value of enzyme activity was little and nugatory which could be explained on the fact that since all the propagules are in same developmental stage, *i.e.*, multiplication stage, and activity of the peroxidase depends on developmental stage which generally increases during rooting and hardening stage (Zeng *et al.*, 2015). When compared with the control plant, the activity of peroxidase was found to be almost similar which shows that addition of plant extract does not evoke production of reactive oxygen molecules. Similar observation was recorded by Sağlam (2013) in cucumber, where exogenous supply of Brassinosteroids (a group novel plant growth substances, initially extracted from pollen grains of rapeseed) did not affect the peroxidase activity. IAA-oxidase is considered to be responsible for the enzymatic oxidative decarboxylation of IAA and the activity of this group of enzymes has been largely associated with adventitious rooting (Porfirio *et al.*, 2016). Similar to peroxidase, IAA-oxidase activity was also found to be almost similar in rose micro-propagules grown on different type and concentration of plant extracts (Fig. 2). Addition of plant extract, after autoclaving the medium (PoA) did not exert any significant effect in both peroxidase and IAA-oxidase activity in rose micro-propagules (data not shown). The results also confirms that the added plant extract do not contain any inhibitory molecules which interfere in activities of these enzymes.

Generally culture media are frequently supplemented with a range of organic extracts like protein hydrolysates, coconut milk, yeast and malt extract, ground banana, orange juice, potato extract, and tomato juice (Molnar *et al.*, 2011). Several natural cytokinin like zeatin, zeatin riboside and C-3 and cell division activity of these growth regulators has already been reported in sweet corn extract. In case of *Anthurium cubense*, replacement

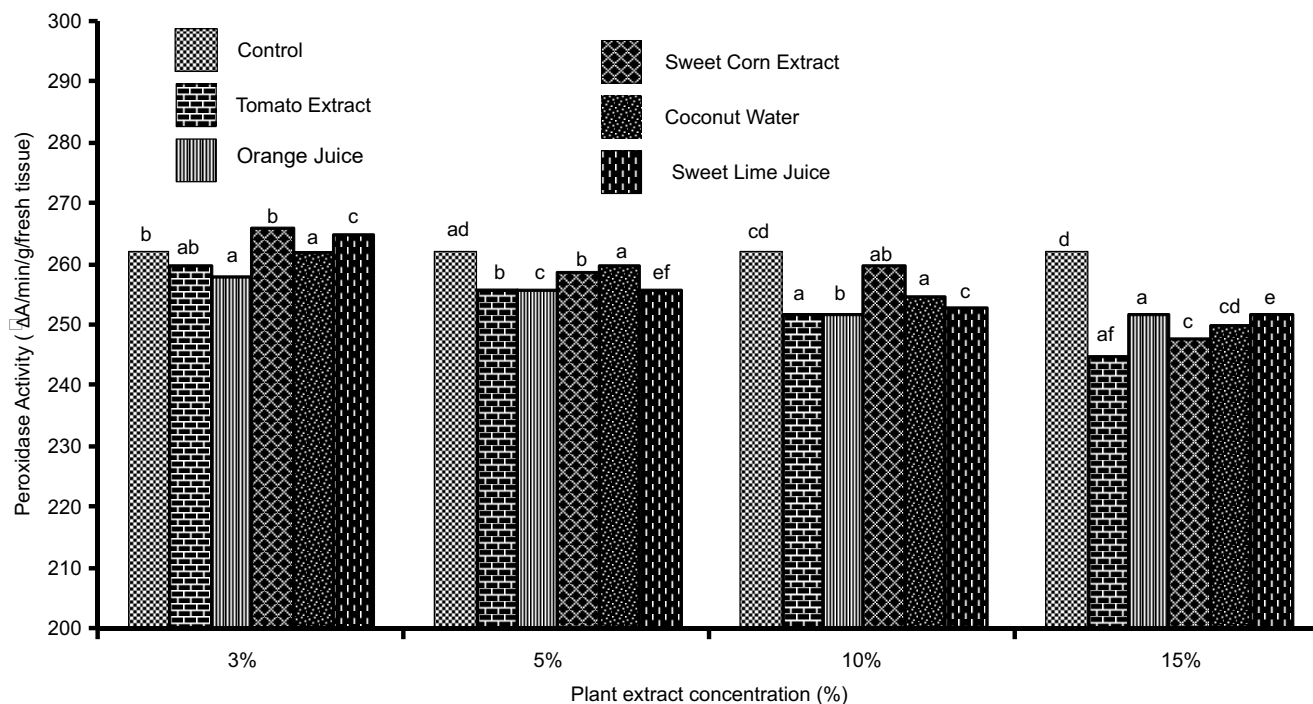


Fig. 1. Activity of peroxidase enzyme in micro-propagules of rose grown *in vitro* on the standard MS medium supplemented with various plant extracts. Extracts were added prior to autoclaving the medium (PrA). Letters indicate significant difference by Tukey's Test ($P < 0.05$).

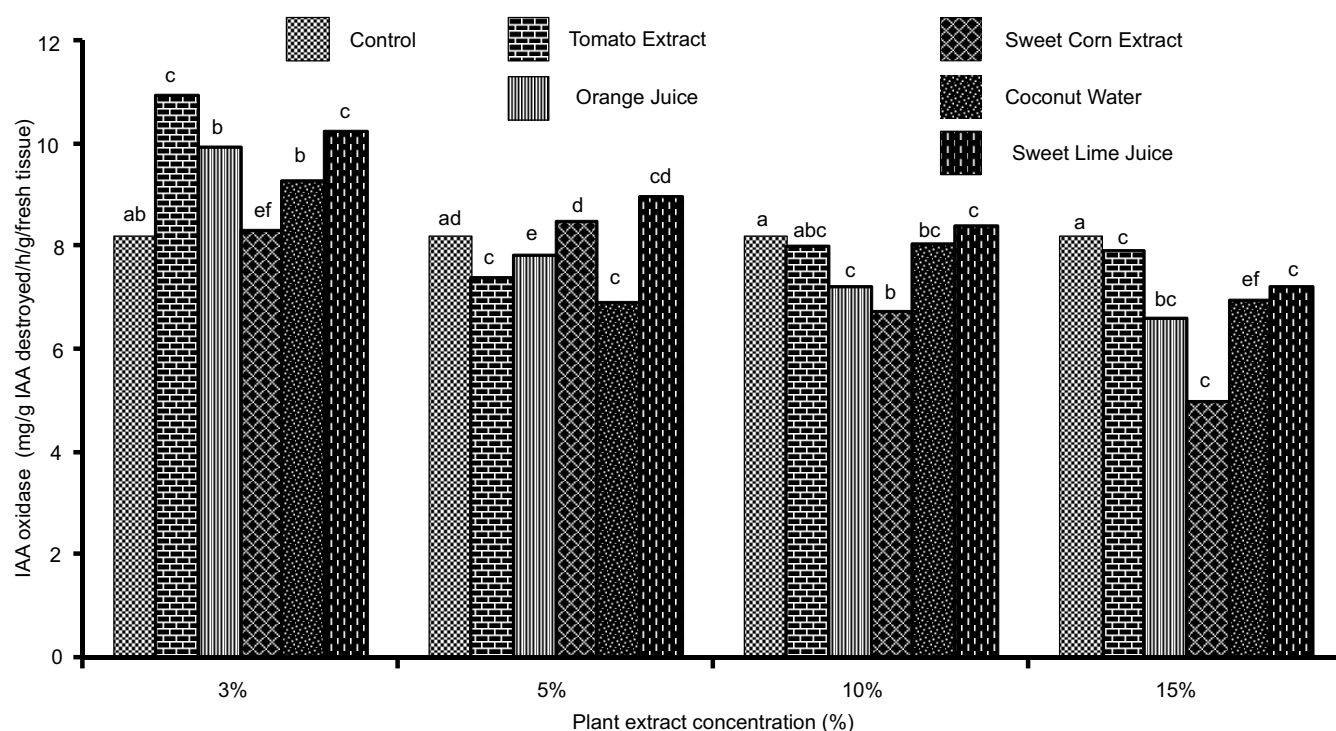


Fig. 2 Activity of IAA-oxidase enzyme in micro-propagules of rose grown *in vitro* on the standard MS medium supplemented with various plant extracts. Extracts were added prior to autoclaving the medium (PrA) Letters indicate significant difference by Tukey's Test ($P < 0.05$).

of cytokinin with cheaper citrus fruit rind derived substance, Pectimorf proved better *in vitro* growth and 90 % survival of plantlets during acclimatization (Montes *et al.*, 2000). Similarly, at 10 mg L⁻¹ concentration, Pectimorf gave *in vitro* multiplication rate of *Spathiphyllum* comparable to 0.5 mg L⁻¹ BA concentration (Hernandez *et al.*, 2009). Similar reports of application of orange juice (@ 10 % v/v) in culturing explants of lemon, grapefruit, sweet orange and mandarin are available (Duran-vila, 1989). Growth promotory role of coconut water in embryo culture of many species was reported long back during early development of plant tissue culture. Later it was discovered that coconut water contains rebosyl-zeatin, which is very similar to zeatin isolated from young maize endosperm (Letham, 1974). Similarly, several growth substances and volatile compounds in sweet lime juice (Maria *et al.*, 2012) and tomato fruit extracts (Rath, 2013) have been reported. Plant growth-promoting substances such as auxins, cytokinins, and betaines have also been reported in seaweed (Khan *et al.*, 2009) and their application as cheaper option in growth of tomato seedling has also been studied (Hernández-Herrera *et al.*, 2014).

Cost analysis and comparison of different plant extract and standard synthetic cytokinin has been depicted in Table 4. It was concluded that replacement of commercial synthetic BA (Sigma-Aldrich) with natural plant extract (@ 5 % v/v) can reduce the cost up to 99 % in reference to cost of PGR. This profit gets amplified with subsequent sub-culturing cycles. There are few reports where natural plant extracts were used to replace synthetic PGRs for lowering down the production cost. Vora and Jasrai (2012) reported that fresh juice of sweet-lime (5 % v/v) provided higher rate of multiplication in *in vitro* cultures of banana. Similarly, coconut water in combination of MS salts was successfully used for regeneration of *Celosia* with the aim of lowering the production cost (Daud *et al.*, 2011).

Table 4. Cost analysis of natural substitutes of BA

Component	Concentration (% v/v)	Cost/L media in INR	Decrease in (%) cost in reference to standard synthetic BA
Synthetic PGR- BA*	0.003	Rs. 52.59	-
Sweet lime juice	5	Rs. 0.80	98.47
Tomato fruit extract	5	Rs. 0.25	99.52
Sweet corn	5	Rs. 0.40	99.23
Orange juice	5	Rs. 0.80	98.47
Coconut water	5	Rs. 0.50	99.04

*Sigma-aldrich

In the current study coconut water (10 % v/v), used as substitute for highly priced synthetic BA, was found effective for *in vitro* shoot multiplication of rose while both tomato extract and sweet lime juice showed comparatively lower shoot multiplication. Supplementation of sweet corn and orange juice also showed successful multiplication and growth of propagules compared to negative control. Although this growth did not equate with the response received in propagules grown on MS medium supplemented with synthetic BA but this slight depression in growth can be compromised on the tiff of high reduction of production cost. In conclusion, coconut water (10 % v/v) provided higher rate of multiplication and was cost-effective also (Table 1) as compared to synthetic BA.

Major apprehension of today's plant tissue culture industry is the high cost for production and maintenance of large numbers of cultures and incessantly rising numbers of accessions. Our outcome showed that by replacing synthetic PGR, the cost of the medium could be reduced significantly. However, this needs to be tested for *in vitro* cultures of a range of plants including many more horticultural plants.

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