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Effects of Phyto-Proteins obtained from Roots of *Boerhaavia diffusa* and Leaves of *Clerodendrum aculeatum* on Proliferation of shoots, Rooting of Isolated Shoots of *Citrus aurantifolia* under *in vitro* Conditions and Acclimatization of its Plantlets

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ABSTRACT: *In vitro* studies indicated that addition of phyto-protein to culture medium of plants was beneficial, for instance, during acclimatization; the plants raised in phyto-protein containing medium showed better growth and survival. Though role of phyto-proteins neither appears to be purely antiviral nor it can be categorized hormonal, the beneficial effects even in their absence were carried forward in subsequent subculture. Moreover, presence of phyto-proteins beyond two subcultures was found detrimental to both growth and proliferation of shoots. An experiment was performed to observe the beneficial effects of phyto-proteins obtained from roots of *B. diffusa* and leaves of *C. aculeatum* on shoot proliferation and growth of excised shoot apices of *Citrus aurantifolia*, their *in vitro* rooting and hardening of *in vitro*-raised plantlets.

I. INTRODUCTION

Lime [*Citrus aurantifolia*, family: Rutaceae] is an important fruit crop of the world. Citrus is vulnerable to infection by a number of pathogens, like viruses, viroids, phyto-plasma and spiroplasma. The main reason behind the low productivity of citrus in India, despite the huge orchard area (ca. 3,69,500 hectares) under cultivation, is the high incidence of viral diseases. Citrus tristeza virus (CTV), a member of Closteroviridae family, is aphid transmitted and is most destructive virus for citrus plants in the world (Thind *et al.* 2004, 2005). Citrus ring spot virus (CRSV) is another serious virus infecting citrus plant.

Enormous tissue culture research has been done in citrus on various aspects, but the work of practical value to citrus cultivation is quite limited (Chaturvedi *et al.* 2001). It may be appropriate that complete plants of *C. sinensis*, *C. grandis* and *C. aurantifolia* were first raised from vegetative tissue *in vitro* in India and grown under field conditions to the fruiting stage (Chaturvedi and Mitra 1974; Chaturvedi *et al.* 1979). Furthermore, a breakthrough in citrus research was also achieved in the country by successfully growing shoot meristems of *C. aurantifolia* and *C. sinensis* to yield cultures of proliferating shoots free from citrus viruses, on which a U.S. patent has also been granted (Chaturvedi *et al.* 2002). Such results, if practically implemented will boost the Citrus Industry in the country and lead to promotion of citrus export, which is nil at present (Chaturvedi and Agnihotri 2002).

II. MATERIALS AND METHODS

Plant material

The plants of *Citrus aurantifolia* were raised under tissue culture conditions from shoots (size 10mm) excised from the proliferating shoot cultures maintained in Tissue Culture Laboratory, National Botanical Research Institute, Lucknow. These cultures were originally obtained from nodal stem segment of 8 year old *C. aurantifolia* plants.

All the procedures concerned with culture work were conducted under aseptic conditions in laminar flow cabinet.

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Culture media

Preparation of stock solutions

Analytical grade chemicals (BDH, Merck, Sigma, Qualigens) were used to prepare culture media. The stock solutions of macro and micro nutrients were prepared by dissolving the salts in double distilled water and then solutions were stored at 4°C. The stock solutions of growth hormones were made by dissolving it in respective solvent, i.e. alcohol or KOH, and stored at 0-2°C. The stock solutions were used within the period specified below:

Stock solution	Period in days
Inorganic salts	60
Trace elements	30
Amino acids and vitamins	10
Auxins, cytokinins and other growth substances	7

Na-Fe-EDTA solution was prepared by dissolving 557 mg FeSO₄.7H₂O in 100 ml warm solution containing 745 mg of Na₂EDTA in double distilled water followed by aeration. The stock solutions were checked before use and only clean and clear stock solutions were used.

Compositions of nutrient media

Compositions of nutrient basal media used are given in Table MM1. The media BM1 and BM2; and BM5 are modifications of Murashige and Skoog; White and Knop's medium, respectively (Singh 2001). BM1 was used for growth and proliferation of shoots of *C. aurantifolia* while BM2 was used for their rooting. Plants grown in aseptic conditions were acclimatized and hardened in liquid medium BM5 (Chaturvedi and Mitra 1974).

Table MM1: Compositions of nutrient media used (mg l⁻¹)

Constituents	BM1	BM2	BM5
(NH ₄) ₂ SO ₄	250	100	—
NH ₄ NO ₃	1500	300	500
KNO ₃	1500	500	250
KCl	—	—	—
KH ₂ PO ₄	150	150	125
NaH ₂ PO ₄ .H ₂ O	—	—	—
CaCl ₂ .2H ₂ O	400	100	—
Ca(NO ₃) ₂ .7H ₂ O	—	—	500
MgSO ₄ .7H ₂ O	450	100	250
Na ₂ SO ₄	—	—	—
Na-Fe-EDTA (ml/l)	5	5	3
MnSO ₄ .4H ₂ O	20	20	22.30
ZnSO ₄ .4H ₂ O	8	8	8.60
H ₃ BO ₃	6	6	6.20
KI	0.8	0.8	0.83
Na ₂ MoO ₄ .2H ₂ O	0.2	0.2	0.25
CuSO ₄ .5H ₂ O	0.02	0.02	0.025
CoCl ₂ .6H ₂ O	0.02	0.02	0.025
Thiamine-HCl	0.20	0.2	—
Pyridoxine-HCl	0.1	0.1	—
Nicotinic acid	0.5	0.5	—
Ascorbic acid	10	10	—
m-Inositol	300	—	—
Glycine	3	—	—
Adenine sulphate	15	5	—
Malt extract	500	—	—
Sugar (%)	5	3	—
pH	5.8	5.8	—
Agar (%)	0.75	0.75	—

* pH was adjusted with 0.1N HCl/KOH prior to autoclaving the nutrient media.

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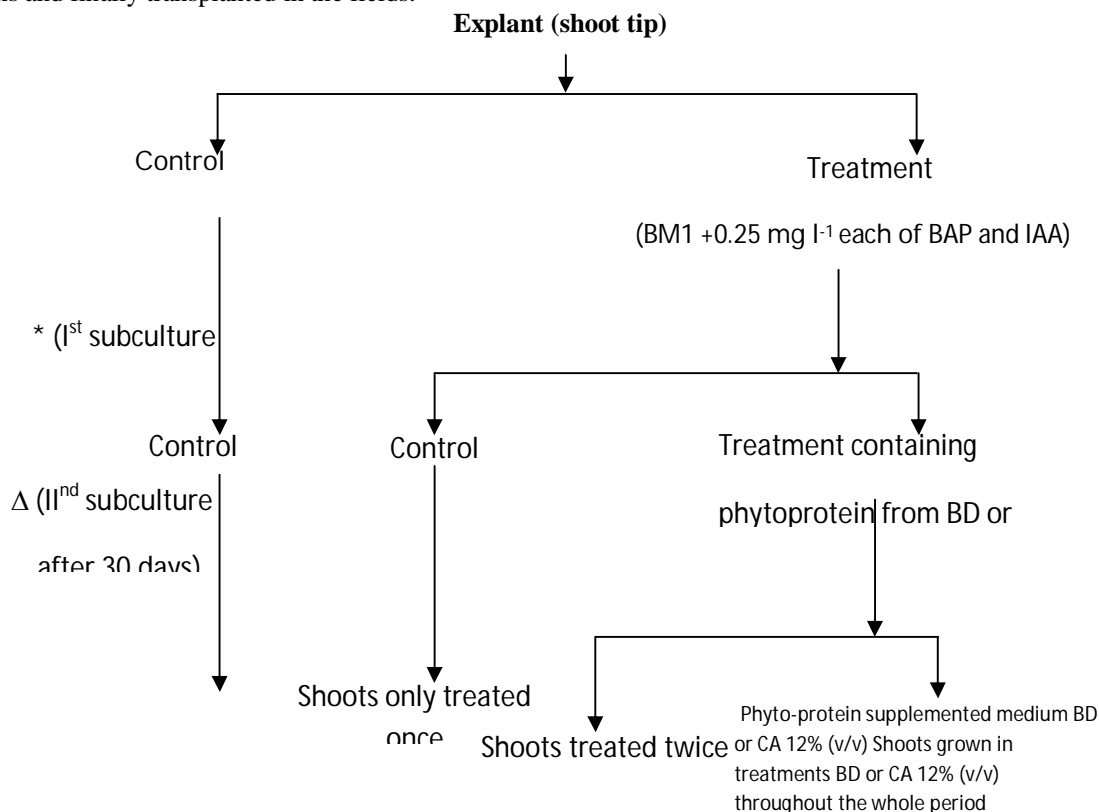
The two phyto-proteins used in culture studies were incorporated into the culture media at concentrations 2, 4, 8, 12, 16 and 20% (v/v) by adding suitable aliquot of original stock preparation.

Sterilization Nutrient media, cultures vessels (test tubes, conical flask), glasswares, like pipettes, glass rods, instruments (forceps, blade, scissors etc.) and distilled water were steam sterilized in an autoclave at 1.08kg/cm² for 15 minutes. Prior to use, instruments were cooled with sterilized single distilled water. The organic compounds like glucose, phytohormones and the phytoprotein of *B. diffusa* and *C. aculeatum* were filter sterilized using bacteria proof filters and added directly to the sterilized media.

Raising of Culture Plants *In-vitro* cultured plantlets were kept at 27 ± 1°C with 70% ± 5% relative humidity and given a photoperiod of 15h. The light intensity of 3000 lux (25µ mol m²s⁻¹ quantum flux density) was obtained from fluorescent tubes supplemented with 40 W incandescent lamps in the culture room.

Hardening of plants *In vitro*-rooted shoots of *C. aurantifolia*. This was followed by rinsing plantlets with distilled water, and then placing them in BM5 medium. These, plants were kept for hardening under aforesaid culture room conditions except that humidity was kept relatively high (90%). This high humidity regime was maintained by covering the plantlets with plastic hoods and spraying water regularly. Humidity was gradually reduced to normal culture room conditions by gradual opening of the windows and finally removing the hood.

The roots of plantlets were washed thoroughly with single distilled water weekly. Small earthen pots containing a mixture of garden soil and farm yard manure in a ratio of 3:1 was used for initial growth of *in vitro*-raised hardened plants. The acclimatized plants were transferred to the soil mixture in pots and kept under identical incubation conditions as used for acclimatization. The potted plants of *C. aurantifolia* were then grown in screen house for about 6 months and finally transplanted in the fields.



* Inoculum for first subculture: 2 bunches of 4-5 shoots each

Δ Inoculum for second subculture: a bunch of 10 shoots

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About 2.5-cm-long shoots of *C. aurantifolia* were excised from the proliferating shoot cultures, i.e., grown throughout in the control medium and those grown in phytoprotein containing treated medium and rooted in agarified nutrient medium BM2 supplemented with 0.25 mg l⁻¹ IBA and 1.0 mg l⁻¹ chlorogenic acid. To see the additional effect of phytoproteins of *B. diffusa* and *C. aculeatum*, these were added at 12% (v/v) to the control rooting medium as well as to the medium containing the same auxin with the phenolic acid.

The *in vitro*-raised plantlets of *C. aurantifolia* were acclimatized in liquid nutrient medium BM5 and the same medium supplemented with *B. diffusa* and *C. aculeatum* phytoproteins 12% (v/v). After one month of hardening, under hardening chamber, the plantlets were transferred to potted soil and were hardened again under glass house conditions.

A summarized account of the steps followed in this experiment is as follows:

1. Shoots grown in the control medium (without any phytoprotein).
2. Shoots grown only once, i.e., at the proliferation stage in the treatment containing phytoprotein (BD/CA).
3. Shoots grown twice in the treatment containing phytoprotein (BD/CA) twice, i.e., for proliferation and rooting.
4. Shoots grown in media supplemented with phytoprotein (BD/CA) from proliferation till hardening, i.e., at the stages, of shoot proliferation, rooting of isolated shoots and hardening of plantlets.

III. RESULTS

Shoot proliferation and shoot growth:

The data regarding the effects of phytoproteins of *B. diffusa* and *C. aculeatum* on shoot proliferation and growth of regenerated shoots of *Citrus aurantifolia* after 30 and 50 days of inoculation in BM1 medium are presented in Plate 4.1, a, b; Table 4.A.1; Plate 4.3, 4.4; Table 4.A.2a; Fig. 4.1, while their proliferation and growth in the medium containing phytoprotein from *C. aculeatum* after 20, 40, 60 days of inoculation given in Plate 4.5; Table 4.A.2b and Fig. 4.2. It was noted that phytoproteins extracted from BD as well as from CA had beneficial effects on growth of offshoots as well as their proliferation. However, the phytoprotein from CA was more efficacious in both of these respects than the phytoprotein from BD. Even at the concentration of 4%, the phytoprotein of BD and 2%, that of CA improved growth and proliferation of shoots, which progressively improved with the increase in the concentration of the phytoprotein to 8, 12 and 16% (v/v) in case of BD and 4, 8 and 16% (v/v) in case of CA, whereas any further increase beyond 16% (v/v) in both the cases i.e. 20% (v/v), proved deleterious to plant growth. Furthermore, the beneficial effects of phytoproteins from both sets of plants showed a carry forward beneficial effects during subcultures, which was more prominent when the 1st subculture was done after 50 days in the control medium and the medium supplemented with phytoproteins, albeit the growth and proliferation of shoots were better in the presence of phytoproteins, where the phytoprotein-nurtured shoots were used as explants. Thus, even beyond 50 days of culture, the phytoproteins had growth enhancing effects which was apparent during subsequent cultures in the control medium (Plate 4.1,c; Table 4.B.1; Plate 4.6,a; Table 4.B.2). It may be inferred that the effect of phytoproteins in the medium was more than of instant nutrition during the culture period and compared with the effects of hormones, as seen by their carry forward effects on enhancement of growth and proliferation of shoots even in the medium devoid of phytoproteins.

In both the cases, the growth and proliferation of the shoots were remarkably far more as compared to the control. Since effects of phytoproteins from CA on growth and proliferation of shoots were spectacular, their effects between the optimum concentration of 16% (v/v) and the next best 8% (v/v) as found in the previous experiment, were studied. It was then observed that the plant extracts had a quantitative role on growth and proliferation of the shoots. It was interesting to note that growth promoting effect of phytoproteins was different than the nutritional factors/components present in the medium, since the shoot growth and proliferation progressively increased up to two subcultures of 30 days each after the initial culture of 50 days, beyond which the presence of phytoproteins in the medium during further subculture was found to be inhibitory for both growth and proliferation of shoots (Plate 4.1,d; Table 4.C.1; Plate 4.6,b; Table 4.C.2).

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It further proved the cumulative effect of phytoproteins on plant growth and development during successive subcultures as hormones, like, cytokinins or auxins, requirement of which gradually declines during successive subcultures in most of the plants. However, the effect of phytoproteins appears to be different from growth hormones as much as their presence becomes inhibitory to plant growth after a few subcultures made in its presence.

Rooting of isolated shoots

In addition to promotive effects of the phytoproteins from BD and CA on shoot growth and proliferation in culture, they also had a beneficial role to play only on growth of the isolated shoots of *C. aurantifolia* when they were rooted. The rooted shoots which were previously nurtured in the presence of phytoproteins from BD or CA showed remarkably better growth than the control shoots, which were not nurtured in phytoproteins (Plate 4.2,a; Table 4.D.1; Plate 4.7; Table 4.D.2). Here also, the phytoproteins from CA were more efficacious than those from BD (Plate 4.9).

Acclimatization of *in vitro*-raised plantlets The rooted shoots being previously nurtured in phytoproteins from BD or CA showed better growth during acclimatization (Plate 4.2,b; Table 4.E.1; Plate 4.8,a; Table 4.E.2) as well as subsequently in the potted soil as compared to the control shoots, which were cultured in the medium devoid of the phytoproteins *ab initio*. Also, the control rooted shoots showed poor *ex vitro* growth than those, which had come from cultures of proliferating shoots grown in the presence of phytoproteins. Such a difference in performance of potted plants vis-à-vis control was more obvious in case of shoots nurtured in phytoproteins from CA than from BD (Plate 4.2,c; Table 4.F.1; Plate 4.8,b; Table 4.F.2)

IV. DISCUSSION

It is interesting to note that the phytoproteins from *B. diffusa* as well as *C. aculeatum* had an additive effect in further promotion of growth and proliferation of isolated shoots of *Citrus aurantifolia* over that already obtained in the optimum treatments containing cytokinin and auxin along with other nutrients and growth promoting substances, including vitamins, amino acid and sucrose (Chaturvedi and Singh 2001). Furthermore, the phytoprotein had the maximum beneficial effect at their optimum concentrations of 12% (v/v) of BD as well as of CA and become toxic at supra optimal concentration of 20% (v/v). If it were only nutritional role of phytoproteins in the medium then the growth and proliferation of shoots should have been promoted in the continuous presence of the phytoproteins from *B. diffusa* and *C. aculeatum* in the medium at their optimum concentration of 12% (v/v) but it was not shown to be so, since their presence in the nutrient medium beyond two subcultures became deleterious for the growth and proliferation of excised shoots.

The shoots nurtured in the presence of phytoproteins did require compulsory cytokinin and auxin in the medium. Thus, the phytoproteins did not substitute for growth hormones. However, at the same time the phytoproteins showed their carry-forward effect on the growth and proliferation which is closely similar to action of growth hormones. Previously there were several reports of phytoproteins extracted from *B. diffusa* as well as from *C. aculeatum* to impart resistance to viral infection in plants, which were otherwise susceptible to the viral infection (Verma *et al.* 1969, 1971, 1979; Awasthi *et al.* 1984; Verma *et al.* 1984, 1985, 1996, 1998, 1999; Gupta *et al.* 2004 and Srivastava *et al.* 2004; Singh *et al.* 2004; Singh and Awasthi 2004; Awasthi *et al.* 2006).

The carry-forward effects of phytoproteins observed in the present investigation in respect of growth and proliferation of shoots is parallel to resistance to viral infection imparted by such phytoproteins for a particular period of the time and gradually fading out later on. However, the presence of such phytoproteins in the plant system after their application has been partially of systemic nature in as much as the resistance to infection pervaded throughout the whole plant body while the phytoproteins extract was applied only at the basal leaves of the plant body (Verma *et al.* 1979; Verma and Awasthi 1980; Verma and Srivastava 1985; Prasad 1988; Verma and Prasad 1992; Verma and Varsha 1995).

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Such effect is homologous to their beneficial influence on the growth and proliferation of excised shoots cultured *in vitro* as the shoots excised from cultures of proliferating shoots grown in the presence of the phytoproteins not only showed better growth in subsequent cultures compared to shoots taken from control, i.e., those grown in the absence of phytoproteins. Also they had better growth in the rooting medium and subsequently in soil as compared to control shoots.

The healthier plants raised from proliferating shoot cultures grown in the presence of the phyto-proteins of *B. diffusa* and *C. aculeatum* may be expected to show resistance to citrus viruses when grown in field conditions and may form better crown, which may lead to production of more fruits than the control. However, at the present stage of investigation increased rate of clonal multiplication of *C. aurantifolia* and production of its healthier plants *ex vitro* with the use of phyto-proteins in the culture medium have been demonstrated. It was seen that phyto-protein from *Boerhaavia diffusa* or *Clerodendrum aculeatum* when added in the nutrient media, promoted the vegetative growth as well as the virus resistance capability of the plantlets grown in that media. The constituents of basal nutrient media (i.e., macro-, micro-, nutrients, trace elements, vitamins and carbon source) affect the overall growth of tissue grown in that media and at their optimum concentrations improved the growth and differentiation of tissues of shoots. Further to the vegetative growth, these substances have also been demonstrated to affect biosynthesis of secondary metabolites in the cultured tissues and organs (Tal and Goldberg 1982; Nakagawa *et al.* 1986; Laukkanen *et al.* 1997; Halaweish *et al.* 1998). Two basal nutrient media, BM3 and BM4 were used, which differed mainly in concentration of KNO_3 , $\text{Ca}(\text{NO}_3)_2 \cdot 7\text{H}_2\text{O}$ and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ from 80, 200 and 360 mg/l to 250, 500 and 250 mg/l, respectively, besides the addition of NH_4NO_3 500 mg/l and KH_2PO_4 125 mg/l in BM4 medium.

Effect of phyto-proteins from *B. diffusa* and *C. aculeatum* on virus resistance capability and vegetative growth of cultured tissue was also seen by Verma *et al.* (1998), Gupta (1999), Srivastava (1999). Effect of some chemical and biological constituents on production of some secondary metabolites, which include phenols imparting to disease resistance, have been studied on some plants like, *Phytolacca americana* (Misawa *et al.* 1975), *Agrostemma githago* (Takayama *et al.* 1987) and *Mirabilis jalapa* (Ikeda *et al.* 1987, 1988). When added to the culture nutrient medium, effect of *B. diffusa* and *C. aculeatum* phytoproteins on vegetative growth as well as on systemic resistance inducing activity against viruses has already been reported under field and green house conditions. It has been shown that these two phytoproteins are highly potent in enhancing the vegetative growth of crops like tomato, okra, brinjal, chilli, mungbean, bottle gourd and ginger, etc. (Verma *et al.* 1979, 1980, 1982, 1984; Awasthi *et al.* 1984; Verma *et al.* 1985, 1995a,b, 1999). Also, the phytoproteins have been demonstrated to enhance the systemic resistance of otherwise susceptible plants, like, tobacco, guar, Bottle gourd and sunnhemp against a number of plant viruses when sprayed on test plants (Verma *et al.* 1982, 1983b, 1984; Verma and Prasad 1983, 1995, 1998).

At a present stage of investigation of phytoproteins from *B. diffusa*, *C. aculeatum* and many other plant spp. listed above do have useful role to play in enhancing growth as well as virus resistance in certain plants. In this way it may be possible to create virus resistant plant without resorting to preying somatic hybridization or transgenesis. In any case, such studies provide ample scope for further investigations which can be utilized for improvement of plant spp. to increase the useful products from them, be their fruit yield or active principles.

V. CONCLUSION

Phyto-proteins from BD as well as CA were beneficial for growth and proliferation of isolated shoots of *C. aurantifolia*, where the latter was more efficacious than the former. The role of phytoproteins was neither purely nutritional nor hormonal. While the growth and proliferation of shoots improved in their respective optimum concentrations up to two subcultures and their beneficial effects were carried forward in the subsequent subcultures even in their absence.

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Table 4.A.1: Effect of different concentrations of phytoprotein, obtained from roots of *Boerhaavia diffusa*, on *in vitro* growth and proliferation of shoot tips (ca. 10 mm in length) of *Citrus aurantifolia* in agarified nutrient medium BM1 supplemented with 0.25 mg l⁻¹ each of BAP and IAA

Treatment	Response*							
	Incubation time (in days)							
	30				50			
Concentration of phytoprotein (v/v)	Length of main shoots (mm)	Number of leaves	Number of off shoots	General condition	Length of main shoots (mm)	Number of leaves	Number of off shoots	General condition
Control (%)	17.1±0.85	5.2±0.51	1.8±0.52	++	20.1±0.74	5.4±0.68	2.5±0.51	+++
2%	17.3±0.75	5.2±0.62	1.8±0.42	++	21.0±0.81	5.6±0.75	2.6±0.62	+++
4%	17.7±0.92	5.4±0.67	1.9±0.62	++	21.9±2.16	6.0±0.79	2.8±0.63	+++
8%	19.6±0.78	5.6±0.59	2.4±0.69	+++	23.7±1.14	6.2±0.63	3.4±0.51	++++
12%	20.9±1.11	6.0±0.75	2.6±0.48	++++	25.2±0.87	6.6±0.69	3.7±0.67	++++
16%	20.5±1.06	5.8±0.51	2.3±0.50	+++	24.6±1.09	6.4±0.72	3.3±0.60	++++

* Data based on average of 20 replicate cultures ± SEM; where number of '+' sign denotes intensity of response

Phytoprotein from *B. diffusa* at 20% concentration had cytotoxic effects (Necrosis of shoot tip and shoot leading to death)

Table 4.A.2(a): Effect of different concentrations of phytoprotein obtained from leaves of *Clerodendrum aculeatum* on *in vitro* growth and proliferation of shoot tips (ca. 10 mm in length) of *Citrus aurantifolia* in agarified nutrient medium BM1 supplemented with 0.25 mg l⁻¹ each of BAP and IAA

Treatment	Response*							
	Incubation time (in days)							
	30				50			
Concentration of phytoprotein (v/v)	Length of main shoots (mm)	Number of leaves	Number of off shoots	General condition	Length of main shoots (mm)	Number of leaves	Number of off shoots	General condition
Control (%)	17.8±1.25	5.1±0.70	1.6±0.42	+	20.9±1.26	5.4±0.70	2.2±0.59	++
2%	18.6±1.46	5.2±0.81	1.9±0.51	+	22.0±1.42	5.8±0.74	2.8±0.68	++
4%	19.9±1.29	5.4±0.65	2.2±0.64	++	23.9±1.33	6.2±0.85	3.3±0.59	+++
8%	21.5±1.07	6.1±0.75	2.7±0.67	+++	26.2±1.39	7.5±0.82	4.0±0.83	++++
16%	22.8±1.90	6.2±1.08	2.4±0.5	++++	29.6±2.07	7.8±1.01	3.5±0.80	++++

* Data based on average of 20 replicate cultures ± SEM; where number of '+' sign denotes intensity of response

Table 4.A.2 (b): Effect of different concentrations of phytoprotein obtained from leaves of *Clerodendrum aculeatum* on *in vitro* growth and proliferation of shoot tips (ca. 10 mm in length) of *Citrus aurantifolia* in agarified nutrient medium BM1 supplemented with 0.25 mg l⁻¹ each of BAP and IAA

Treatment	Response*											
	Incubation period 60 days											
	20				40				60			
Concentration of phytoprotein (v/v)	Length of main shoots (mm)	Number of leaves	Number of off shoots	General condition	Length of main shoots (mm)	Number of leaves	Number of off shoots	General condition	Length of main shoots (mm)	Number of leaves	Number of off shoots	General condition
0% (Control)	13.7±0.94	3.5±0.52	1.2±0.51	+	18.2±1.32	5.3±0.82	2.1±0.57	++	21.4±1.44	5.8±0.73	2.6±0.67	++
8%	15.8±1.03	3.8±0.78	1.5±0.57	++	23.1±1.19	5.6±0.97	3.1±0.73	+++	28.6±1.34	6.7±0.94	4.3±0.69	++++
12%	16.8±1.22	4.2±0.51	1.8±0.78	+++	26.1±1.37	6.3±0.74	3.4±0.79	++++	30.8±1.39	7.3±0.94	4.7±0.95	++++
16%	16.6±1.07	4.1±0.48	1.3±0.48	+++	26.0±1.17	6.1±0.67	2.6±0.51	++++	30.2±1.47	7.1±0.97	3.6±0.69	++++

* Data based on average of 20 replicate cultures ± SEM; where number of '+' sign denotes intensity of response

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Pytoprotein from *C. aculeatum* at 20% concentration had cytotoxic effects (Necrosis of shoot tip and shoot leading to death)

Table 4.B.1: Effect of growth promoting phyto-protein 12% (v/v) obtained from roots of *Boerhaavia diffusa* on proliferation and growth of regenerated shoots of *Citrus aurantifolia* during first sub culturing (two bunch of 4-5 shoots was inoculated each with average length of about 20mm) in agarified nutrient medium BM₁ supplemented with 0.25 mg l⁻¹ each of BAP and IAA

Treatment	Response*		
	Incubation 30 days		
	Length of main shoot (mm)	Number of proliferating shoots	General condition
Non treated shoots transferred to BM ₁ medium (Control)	21.0±1.05	18.5±1.07	+
Treated shoots transferred to BM ₁ medium	23.9±0.87	21.4±1.26	++
Treated shoots transferred to BM ₁ medium with phytoprotein	26.8±0.79	23.7±1.05	+++

* Data based on average of 10 replicate cultures ± SEM; where number of '+' sign denotes intensity of response

Table 4.B.2: Effect of phyto-protein 12% (v/v) obtained from leaves of *Clerodendrum aculeatum* on proliferation and growth of regenerated shoots of *Citrus aurantifolia* during first subculturing (inoculated two bunch of 4-5 shoots each with average length of about 20 mm) in agarified nutrient medium BM₁ supplemented with 0.25 mg l⁻¹ each of BAP and IAA

Treatment	Response*		
	Incubation 30 days		
	Length of main shoot (mm)	Number of proliferating shoots	General condition
Non treated shoots transferred to BM ₁ medium (Control)	22.1±0.87	19.0±0.81	++
Treated shoots transferred to BM ₁ medium	25.5±1.08	23.5±1.58	+++
Treated shoots transferred to BM ₁ medium with phytoprotein	29.2±0.82	26.0±1.42	++++

* Data based on average of 10 replicate cultures ± SEM; where number of '+' sign denotes intensity of response

Table 4.C.1. Effect of phytoprotein 12% (v/v) obtained from roots of *Boerhaavia diffusa* on proliferation and growth of regenerated shoots of *Citrus aurantifolia* during second subculturing (inoculated about 10 shoots with average length of about 20 mm) in agarified nutrient medium BM₁ supplemented with 0.25 mg l⁻¹ each of BAP and IAA

Treatment	Response*		
	Incubation 30days		
	Length of main shoot (mm)	Number of proliferating shoots	General condition
Non treated shoots transferred to BM ₁ medium (Control)	22.5±1.08	16.5±1.06	++
Treated shoots once transferred to BM ₁ medium	25.5±0.52	18.8±0.91	+++
Treated shoots twice transferred to BM ₁ medium	28.1±0.73	21.2±1.05	++++
Treated shoots twice transferred to BM ₁ medium with phytoprotein	30.1±0.75	23.1±1.11	+++++

* Data based on average of 10 replicate cultures ± SEM; where number of '+' signs denotes intensity of response

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Table 4.C.2: Effect of phytoprotein 12% (v/v) obtained from leaves of *Clerodendrum aculeatum*, on proliferation and growth of regenerated shoots of *Citrus aurantifolia* during second subculturing (inoculated about 10 shoots each with average length of about 20 mm) in agarified nutrient medium BM1 supplemented with 0.25 mg l⁻¹ each of BAP and IAA

Treatment	Response*		
	Incubation 30 days		
	Length of main shoot (mm)	Number of proliferating shoots	General condition
Non treated shoots transferred to BM1 medium (Control)	22.7±1.16	18.3±1.89	++
Treated shoots once transferred to BM1 medium	26.4±1.18	20.1±1.52	+++
Treated shoots twice transferred to BM1 medium	29.1±1.45	23.4±1.17	++++
Treated shoots twice transferred to BM1 medium with phytoprotein	32.1±0.74	24.7±1.34	+++++

* Data based on average of 10 replicate cultures ± SEM; where number of '+' sign denotes intensity of response

Table 4.D.1: Effect of phytoprotein, 12% (v/v) obtained from roots of *Boerhaavia diffusa* on rooting of isolated shoots (ca. 25 mm in length) and growth of rooted plantlets of *Citrus aurantifolia* under *in vitro* conditions in agarified nutrient medium BM2 containing 0.25 IBA and 1.0 Chlorogenic acid (mg l⁻¹)

Treatment	Response*		
	Incubation 30 days		
	Length of main shoot (mm)	Number of leaves	General condition of plantlets
Non treated shoots transferred to BM2 medium (control)	34.9±1.41	5.9±0.71	++
Treated shoots transferred to BM2 medium	37.7±1.75	6.4±0.68	+++
Treated shoots transferred to BM2 medium with phytoprotein	40.2±1.66	7.1±0.64	++++

* Data based on average of 20 replicate cultures ± SEM; where number of '+' sign denotes intensity of response

Table 4.D.2: Effect of phytoprotein 12% (v/v) obtained from leaves of *Clerodendrum aculeatum* on rooting of isolated shoots (ca. 25 mm in length) and growth of rooted plantlets of *Citrus aurantifolia* in *in vitro* conditions in agarified nutrient medium BM2 supplemented with 0.25 IBA and 1.0 chlorogenic acid (mg l⁻¹).

Treatment	Response*		
	Incubation 30 days		
	Length of main shoot (mm)	Number of leaves	General condition of plantlets
Non treated shoots transferred to BM2 medium (control)	34.3±1.75	6.5±0.76	++
Treated shoots transferred to BM2 medium	39.8±2.00	6.9±0.78	+++
Treated shoots transferred to BM2 medium with phytoprotein	47.4±1.96	8.0±0.75	++++

* Data based on average of 20 replicate cultures ± SEM; where number of '+' sign denotes intensity of response

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Table 4.E.1: Effect of phyto-protein, 12% (v/v) obtained from roots of *Boerhaavia diffusa* on growth of *in vitro* rooted plantlets of *Citrus aurantifolia* during acclimatization in liquid nutrient medium BM5

Treatment	Response*		
	Incubation 30 days		
	Length of main shoot (mm)	Number of leaves	General condition of plantlets
Non treated plantlets BM5 medium (Control)	47.7±1.70	6.5±1.70	+
Treated plantlets once transfer to BM5 medium	50.2±1.62	6.8±0.63	++
Treated plantlets twice transfer to BM5 medium	52.0±1.74	7.4±0.51	+++
Treated plantlets twice transfer to BM5 medium with phytoprotein	55.6±1.42	7.6±0.51	++++

* Data based on average of 20 replicate plantlets ± SEM; where number of '+' sign denotes intensity of response

Table 4.E.2: Effect of phytoprotein 12% (v/v) obtained from leaves of *Clerodendrum aculeatum* on growth of *in vitro* rooted plantlets of *Citrus aurantifolia* during acclimatization in liquid nutrient medium BM5

Treatment	Response*		
	Incubation 30 days		
	Length of main shoot (mm)	Number of leaves	General condition of plantlets
Non treated plantlets BM5 medium (Control)	44.3±1.15	6.3±0.82	++
Treated plantlets once transfer to BM5 medium	51.6±2.11	7.2±0.78	+++
Treated plantlets twice transfer to BM5 medium	56.4±1.42	7.4±0.69	++++
Treated plantlets twice transfer to BM5 medium with phytoprotein	65.4±2.11	8.2±0.78	+++++

* Data based on average of 20 replicate plantlets ± SEM; where number of '+' signs denotes intensity of response

Table 4.F.1: Growth of non treated and treated (with *Boerhaavia diffusa* phytoprotein, 12% v/v) *in vitro*-raised plantlets of *Citrus aurantifolia* in potted soil.

Treatments	Response*			
	Incubation 30 days			
	Length of main shoot (mm)	Number of leaves	Leaf area (Max length X Max breadth) (mm)	General condition of plantlets
Non treated plantlets (Control)	73.0±1.70	7.6±0.51	44.7±2.31×28.7±1.25	++
Plantlets treated once with phytoprotein	74.8±1.47	7.8±0.63	48.9±2.29×29.1±0.87	+++
Plantlets treated twice with phytoprotein	77.3±2.05	8.1±0.73	51.0±0.94×30.0±0.67	+++
Plantlets treated with phytoprotein throughout whole period	83.9±1.85	8.5±0.53	52.3±1.34×31.2±0.63	++++

* Data based on average of 20 replicate plants ± SEM; where number of '+' sign denotes intensity of response

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Table 4.F.2: Growth of non-treated and treated (with *Clerodendrum aculeatum* phytoprotein 12% v/v) *in vitro* raised plantlets of *Citrus aurantifolia* in potted soil.

Treatment	Response*			
	Duration (30 days)			
	Length of main shoot (mm)	Number of leaves	Leaf area (Max length X Max breadth) (mm)	General condition of plantlets
Non treated plantlets (Control)	72.3±1.82	7.4±0.51	30.9±0.87×21.0±0.81	++
Plantlets treated once with phytoprotein	75.5±2.35	7.5±0.52	43.4±1.64×27.1±0.87	+++
Plantlets treated twice with phytoprotein	86.7±2.11	8.7±0.94	48.8±0.91×28.9±0.97	++++
Plantlets treated with phytoprotein throughout whole period	99.8±2.62	9.6±0.51	49.3±0.82×33.5±1.35	+++++

* Data based on average of 20 replicate plants ± SEM; where number of '+' sign denotes intensity of response

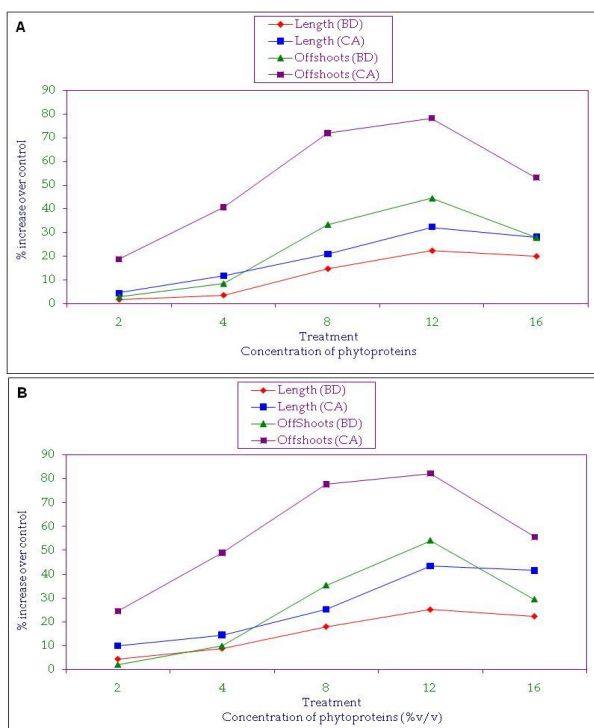


Fig. 4.1 Effect of different concentration of phytoproteins obtained from *Boerhaavia diffusa* (BD) and *Clerodendrum aculeatum* (CA) on shoot length and number of offshoots of *Citrus aurantifolia* under *in vitro* conditions

A. Incubation 30 days

B. Incubation 50 days

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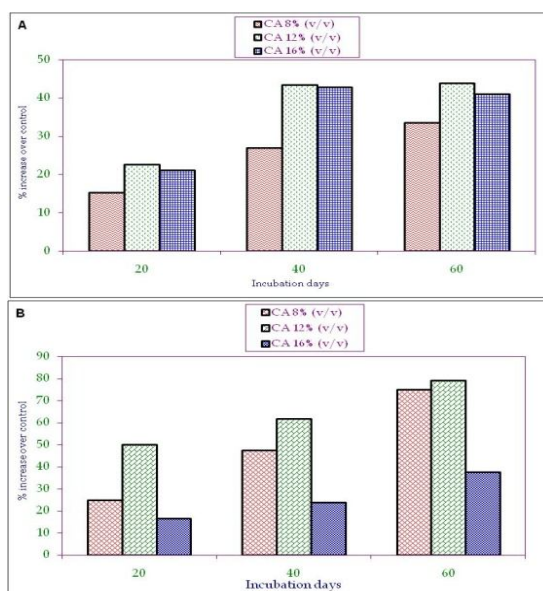


Fig 4.2. Effect of different concentrations of phytoprotein from *Clerodendrum aculeatum*(CA) of *Citrus aurantifolia*
A. Shoot length of *Citrus aurantifolia*
B. Offshoots of *Citrus aurantifolia*.

The shoot proliferation nutrient medium BM1, the standardized nutrient medium for *C. aurantifolia* shoot culture, developed in the Tissue Culture Laboratory, NBRI, Lucknow, comprised a modification of Murashige and Skoog (1962) medium supplemented with 0.25 mg l⁻¹ each of BAP and IAA and was used as the control for this experiment. The phytoproteins of *B. diffusa* and *C. aculeatum*, after filter sterilization, were added to the autoclaved nutrient medium at 2, 4, 8, 12, 16 and 20% (v/v). Healthy shoots of *C. aurantifolia* of uniform size (10 mm in length) with 2-3 leaves were excised from cultures of *in vitro* proliferating shoots. The phytoproteins (12% v/v) of *B. diffusa* and *C. aculeatum* were added to agarified nutrient medium BM1 for proliferation and growth of regenerated shoots of *C. aurantifolia* during the first culture, 2 groups of 4-5 shoots each, of an average length of about 20 mm were inoculated and then subcultured after one month, during which the shoots had attained an average length of about 25 mm. Ten such shoots were inoculated per culture. In the first culture, shoots from phytoprotein, from BD or CA supplemented medium were transferred to the control, i.e., the same medium. After 50 days of subculture, bunches of shoots from the control as well as from the phytoprotein supplemented medium were transferred to the control medium. The procedure is also presented in a flow chart. Number of passages of explants from control medium and from treatment containing phytoproteins from BD or CA, provided has been systematically described as below:

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PLATE- 4.1

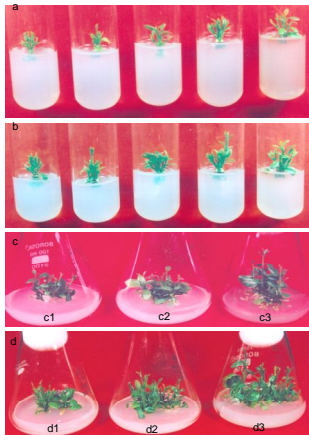


Plate- 4.1 Effect of addition of different concentrations of phytoprotein obtained from roots of *Berhaavia diffusa* on in vitro growth and proliferation of shoot tips (ca. 10 mm in length) of *Citrus aurantifolia* in agarified nutrient medium BM1 supplemented with 0.25 mg/l each of BAP and IAA (left to right: Control, 4%, 8%, 12% and 16% phytoprotein)
a. after 30 days of inoculation, b. after 50 days of inoculation
c. After first subculturing (30 days)
c1. control c2. treated once c3. treated twice (12% phytoprotein)
d. After second subculturing (30 days)
d1. control d2. treated twice d3. treated thrice (12% phytoprotein)

PLATE- 4.2

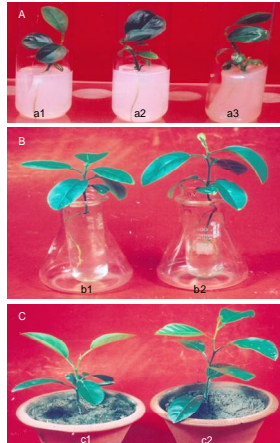


Plate- 4.2 Effect of phytoprotein from *Berhaavia diffusa* on rooting of isolated shoots of *Citrus aurantifolia* under in vitro conditions and acclimatization of its plantlets
A. Addition of phytoprotein (12% v/v) on rooting of isolated shoots (ca. 25 mm in length) and growth of rooted plantlets of *Citrus aurantifolia* in vitro conditions in agarified nutrient medium BM2 supplemented with 0.25 IBA and 1.0 chlorogenic acid mg/l
a1. Control, a2. Treated once, a3. Treated twice
B. Hardening in modified Knop's solution b1. Control b2. Treated thrice
C. Hardening in potted soil c1. Control, c2. Treated through out whole period

PLATE- 4.3

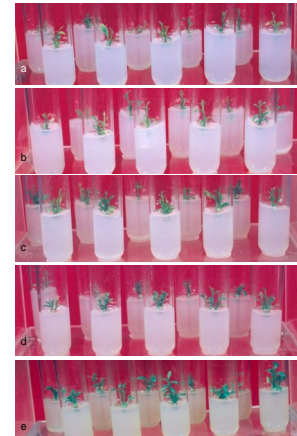


Plate- 4.3 Effect of addition of different concentrations of phytoprotein obtained from leaves of *Clorodendrum azukiatum* on in vitro growth and proliferation of shoot tips (ca. 10 mm in length) of *Citrus aurantifolia* in agarified nutrient medium BM1 supplemented with 0.25 mg/l each of BAP and IAA after 30 days of inoculation
a. Control, b. 2% phytoprotein, c. 4% phytoprotein, d. 8% phytoprotein, e. 16% phytoprotein

PLATE- 4.4

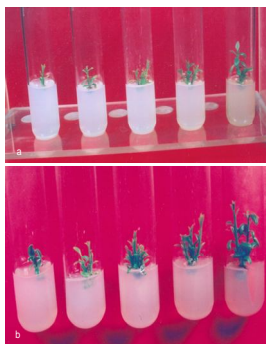


Plate- 4.4 Effect of addition of different concentrations of phytoprotein obtained from leaves of *Clorodendrum azukiatum* on in vitro growth and proliferation of shoot tips (ca. 10 mm in length) of *Citrus aurantifolia* in agarified nutrient medium BM1 supplemented with 0.25 mg/l each of BAP and IAA (left to right: Control, 2%, 4%, 8% and 16% phytoprotein)
a. after 30 days of inoculation b. after 50 days of inoculation

PLATE- 4.5

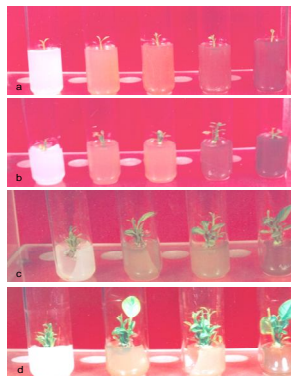


Plate- 4.5 Effect of addition of different concentrations of phytoprotein obtained from leaves of *Clorodendrum azukiatum* on in vitro growth and proliferation of shoot tips (ca. 10 mm in length) of *Citrus aurantifolia* in agarified nutrient medium BM1 supplemented with 0.25 mg/l each of BAP and IAA (left to right: Control, 8%, 12%, 16% and 20% phytoprotein)
a. At the time of inoculation, b. After 20 days of inoculation (20% phytoprotein had cytotoxic effects than necrosis of shoot tip and shoot leading to death).
c. After 40 days of inoculation d. After 60 days of inoculation

PLATE- 4.6

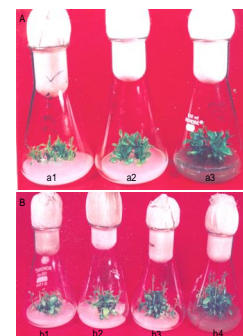


Plate- 4.6 Effect of addition of phytoprotein (12% v/v) obtained from leaves of *Clorodendrum azukiatum* on proliferation and growth of regenerated shoots of *Citrus aurantifolia* in agarified nutrient medium BM1 supplemented with 0.25 mg/l each of BAP and IAA
A. During first subculturing (30 days) B. During second subculturing (30 days)
a1. Control a2. Treated once a3. Treated twice
b1. Control b2. Treated once b3. Treated twice b4. Treated thrice

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PLATE- 4.7

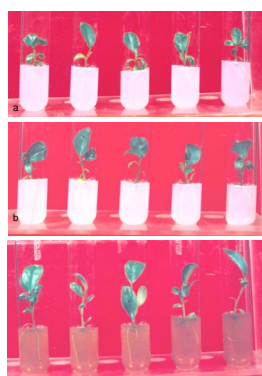


Plate- 4.7 Effect of addition of phytoprotein (12% v/v) obtained from leaves of *Clorodendrum aculatum* on rooting of isolated shoots (ca. 25 mm in length) and growth of rooted plantlets of *Citrus aurantifolia* in vitro in agarified nutrient medium BM2 supplemented with 0.25 mg/l IBA and 1.0 mg/l chlorogenic acid
a. Control b. Treated once c. Treated twice

PLATE- 4.8



Plate- 4.8 Effect of addition of phytoprotein (12% v/v) obtained from leaves of *Clorodendrum aculatum* on growth of in vitro rooted plantlets of *Citrus aurantifolia* during acclimatization
A. In liquid nutrient medium BM5 a1. Control a2. Treated twice a3. Treated thrice
B. Growth of non-treated and treated (with *Clorodendrum aculatum* phytoprotein 12% v/v) in vitro raised plantlets of *Citrus aurantifolia* in potted soil.
b1. Control b2. Treated throughout the whole period

PLATE- 4.9

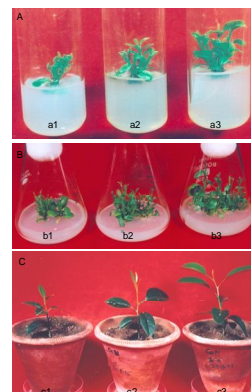


Plate- 4.9 Growth and proliferation inducing activity of optimum concentration of phytoprotein of *Boerhaavia diffusa* (BD) / *Clorodendrum aculatum* (CA) on in vitro raised plantlets of *Citrus aurantifolia*
a1.b1.c1. Control
a2.b2.c2. BD treated
a3.b3.c3. CA treated

On the contrary, their presence in the medium beyond two subcultures was deleterious for both growth and proliferation of shoots. The phyto-protein-nurtured shoots showed better growth in rooting medium, while there was no beneficial effect on the process of rooting. Similarly, such rooted shoots also showed better *ex vitro* growth than the shoots obtained from the control.

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