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Efficient *in vitro* regeneration protocol of *Centella asiatica* (L.) Urban: An endemic and underutilized nutraceutical herb

Kanchan Joshi, Preeti Chaturvedi* and Shubhpriya

Department of Biological Sciences, College of Basic Sciences and Humanities, Pantnagar 263145, India.

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The present communication reports an efficient *in vitro* plantlet regeneration protocol for endemic umbellifer *Centella asiatica* (L.) urban via callus mediated organogenesis from leaf and stem explants. The plant is pharmacologically very important and its consumption as underutilized green leafy vegetable affluent in micronutrients is communally conscientious for its threatened status. Therefore, there is an importunate need to preserve its germplasm so that pharmacologically active constituent can be made available all over the year without causing loss of species from wild. Optimum callusing was observed in MS + benzylaminopurine (BAP, 0.5 mg/l) + α-napthalene acetic acid (NAA, 0.3 mg/l) in both leaf and stem explants with callus induction frequency 75 and 83.33%, respectively. For shooting, MS + BAP (0.5 mg/l) in leaf and MS + BAP (0.75 mg/l) in stem derived callus were found to be most efficient. Rooting of *in vitro* raised shoots was best induced on full strength MS media supplemented with indole- 3- butyric acid (IBA, 0.5 mg/l). The regenerated plants were acclimatized in controlled environment and successfully transferred in field condition displaying normal development.

Key words: *Centella asiatica*, *in vitro*, micropropagation, organogenesis, plant growth regulators, plant regeneration, tissue culture.

INTRODUCTION

Centella belonging to Apiaceae family is a diverse genus encompassing approximately 50 species which include the medicinally eminent plant *Centella asiatica* (L.) Urban (James and Dubery, 2009). The herb is acknowledged by distinct names, for instance Pegaga in Malaysia, Indian Pennywort in Europe, Gotukola in America, Mandookparni in India, Pegagan or Kakikuda in Indonesia, Luiei Gon Gen or Tung Chain in China (Tolkah, 1999). It is a stonoliferous, perennial, slightly aromatic herb flourishes plentifully in moist localities (Fosberg et al., 1979; van Wyk et al., 1997; Whistler, 1988) disseminated all over the world including India, China, Nepal, Madagascar, Srilanka, Indonesia and Southern America (Satake et al., 2007; Schaneberg et al., 2003; Zheng and Quin, 2007). In India, *Centella* escalate up to an elevation of 600 to 1800 m above sea level (Patra et al., 1998). The most eminent bioactive component found in *C. asiatica* are triterpenoid glycosides (asiatic acid, madecassic acid), saponin glycosides (bhramiside, bhraminoside) and flavonoids

*Corresponding author. E-mail: an_priti@yahoo.co.in. Fax: 05944233473.

Abbreviations: BA, 6- benzylaminopurine; **IAA,** indole- 3- acetic acid; **IBA,** indole- 3- butyric acid; **NAA,** α-napthalene acetic acid; **TDZ,** thiodiazuran.

which are known to possess antileprotic, antifilarial, antibacterial, adaptogenic, antifeedant and antiviral properties (Warrier et al., 1994). Extract of the whole plant is reported to have anticancerous activity (Yu et al., 2006) and the methanol extract of aerial parts of the *C. asiatica* inhibits the growth of human uterine carcinoma, human gastric carcinoma and murine melanoma cells *in vitro* (Yoshida et al., 2005). Vellarine, the active principle present in the leaves, is an oleaginous white crystalline substance considered advantageous in cognitive impairment (Bakhru, 2003). Hence, it has been frequently used as medicine in Indian Herbal Pharmacopoeia, the German Homeopathic Pharmacopoeia, European Pharmacopoeia and the Pharmacopoeia of the People's Republic of China (Brinkhaus et al., 2000).

Owing to the high therapeutic potential, it is of high stipulate in pharmaceutical industries which escorts to its overexploitation resulting in the relapsing of the population of *C. asiatica* to a precarious level that a ban on its collection from their natural habitat has been recommended (Nayar and Shastry, 1987). *C. asiatica*, thus recognized as threatened medicinal herb (Sharma and Kumar, 1998; Singh, 1989) is endemic to Western Ghats of South India (Nayar, 1996). Thus, it has become imperative to develop alternative approaches for its conservation, in order to protect it from the verge of extinction. *In vitro* culture technique offers a viable tool for the mass propagation of plant and conservation of rare, threatened and endangered germplasm (Rao, 2004). Therefore, in the present study, an attempt has been made for *in vitro* plantlet regeneration of *C. asiatica* through callus mediated organogenesis.

MATERIALS AND METHODS

Actively growing mature plant of *C. asiatica* (L.) Urban was collected from MRDC, Pantnagar. Firstly, plants were washed under running tap water to make them free from dust and soil particles followed by immersing in 2% mild detergent for 10 to 15 min with recurrent agitation. The plant material was then dipped in Bavistin (1%) for half an hour, rinsed three to four times with sterile water. It was further surface sterilized using 70% ethanol for 30 s, followed by 0.1% $HgCl_2$ for a minute and then again washed with sterile distilled water 3 to 4 times. The pH of the medium was adjusted to 5.8 ± 0.1 using 0.1 N HCL or 0.1 N NaOH solution followed by sterilization for 20 min at 120°C. The cultures were incubated at 25 ± 2°C under 16 h photoperiod and light intensity of 2200 to 4000 lux. For callus induction, sterile explants (leaf and stem) were inoculated aseptically in culture bottles containing 50 ml. MS medium supplemented with different phytohormones in various combinations namely, 2-4 D, IBA, NAA, BAP, TDZ. After 2 to three weeks of culture, calli initiated from the excised leaf and stem explants and were further sub cultured in the fresh medium at regular intervals for continuous growth. For induction of microshoots and its differentiation, calli were transferred to MS medium supplemented with different combinations and concentrations of BAP and Kinetin. For rooting response, excised shoots were transferred to full strength MS basal medium fortified with different concentration and combination of auxins namely, IAA, NAA, 2-4 D and IBA. Complete plantlet regenerated after 4 to 5 weeks on rooting media. The well rooted plantlets were detached cautiously from culture bottle under running tap water in order to remove relics of agar and transferred in plastic pots (Figure 6) containing sterilized vermiculite, sand and soil (1:1:1) and kept under glass house enclosed with translucent polythene bags for a week to maintain humidity (Figure 7). After 25 to 30 days, they were transferred to field conditions.

All the experiments were performed in replicates. For callus induction and shooting, five replicates and for root induction, nine replicates for each hormone combination were employed. Mean values obtained from different treatments were then subjected to one way analysis of variance (Snedecor and Cochran, 1994).

RESULTS AND DISCUSSION

Callogenic response was observed in all the combinations except the media free of growth regulators. The induced calli were green in leaf and creamish green in stem explants (Figures 1 and 2). Optimum response was achieved in hormone combination of BAP (0.5 mg/l) + NAA (0.3 mg/l) in both explants having callus induction frequency 75 and 83.33% and biomass 1.326 and 0.996 g in leaf and stem explants, respectively (Tables 1 and 2). This finding is in accordance with earlier studies on this plant (Banerjee et al., 1999; Deshpande et al., 2010). The differential response of explants to callusing may be attributed due to varying concentration of endogenous levels of auxin and cytokinins which relatively influence the genes to trigger differentiation of cells (Rout and Das, 1997; Thorpe, 1983). Shoot induction occurred within 2 to three weeks of culture of calli. Among the various combinations of phytohormones used, MS + BAP (0.5 mg/l) was found to be optimum for leaf explants with 88.88% shoot induction factor, shoot length 5.6 cm and number of five shoots in 16 days (Figure 3). For stem explants, MS + BAP (0.75 mg/l) was found to be most conducive with 77.77% shoot induction factor, 4.6 cm shoot length and 8number of shoots (Figure 4). Thus, BAP was found to be effective and superior for shoot induction in both explants (Tables 3 and 4). This finding is inconsistence with earlier study on this plant (George et al., 2004). Studies on other medicinal plants (Chirangini et al., 2005; Ghanti et al., 2004; Karthikeyan et al., 2007; Lal et al., 1988) have also validated the efficiency of BAP for shoot induction and proliferation. The efficiency of BAP in shoot induction may be due to the ability of plant tissue to metabolize natural hormones more readily than artificial growth regulators or due to the ability of BAP to induce production of natural hormones namely, zeatin within the tissue and thus, working through natural hormone system (Sharma and Wakhlu, 2003). Alteration in the action of distinctive cytokinin receptors, leaf and stem explants may be attributed to their differential uptake proportions (Blakesey, 1991), diverse translocation rates to meristematic sections and in certain metabolic progression where they fail to respond it may be

Figure 1. Callus induction from leaf explant (a), and callus proliferation from leaf explant (b).

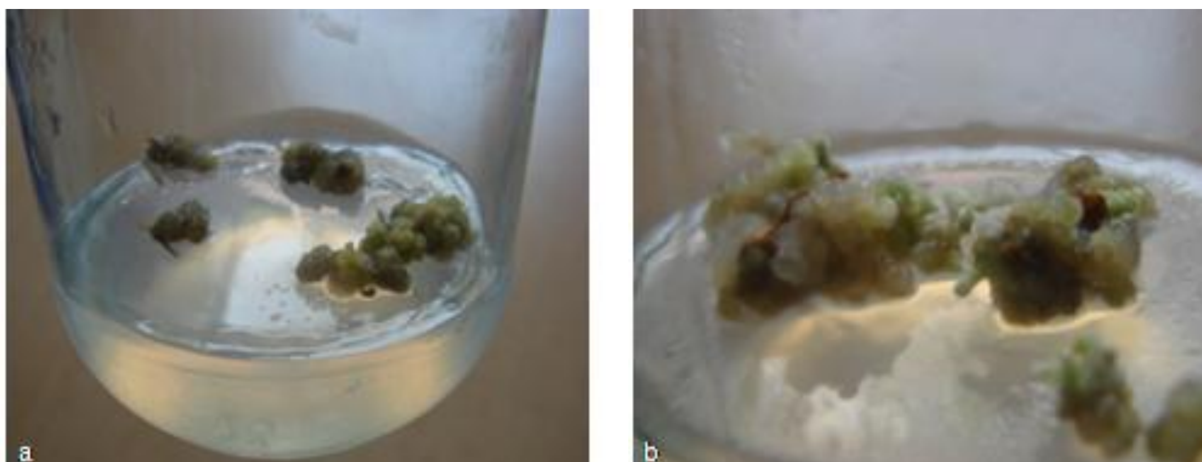

Figure 2. Callus induction from stem explant (a), and callus proliferation from stem explant (b).

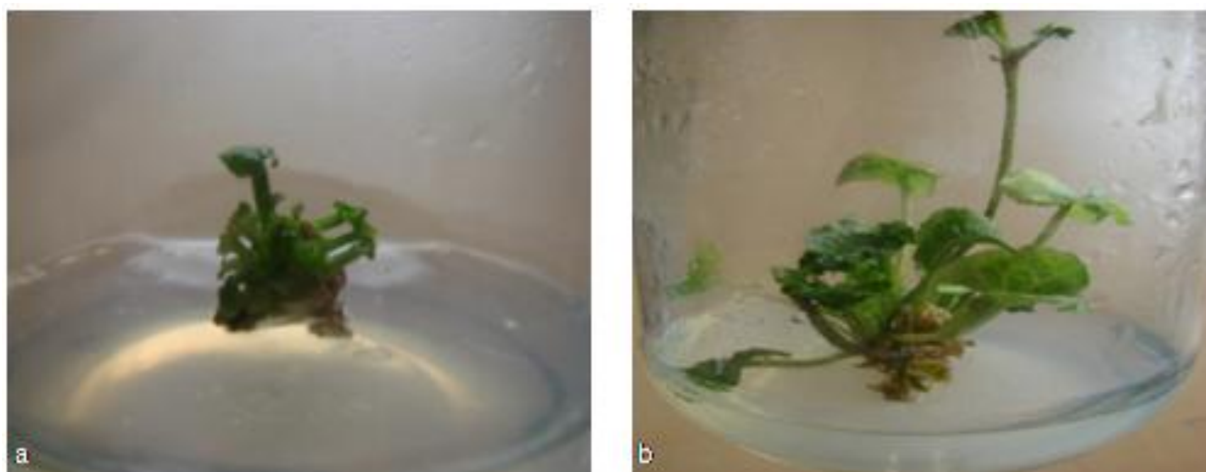

Figure 3. Shoot initiation from leaf explants after 2 to 3 weeks of culture (a), and shoot proliferation from leaf explants after 4 to 5 weeks of culture (b).

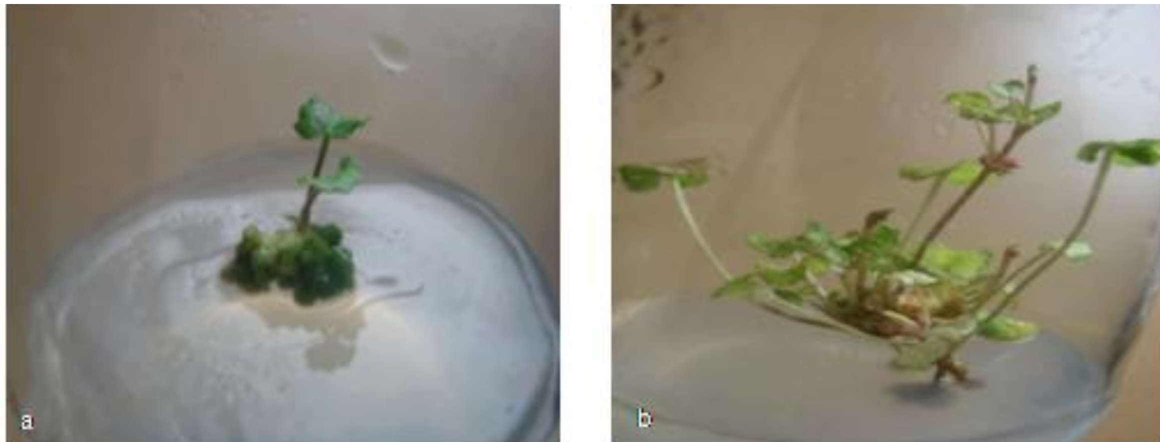

Figure 4. Shoot initiation from stem explants after 2 to 3 weeks of culture (a), and shoot proliferation from stem explants after 4 to 5 weeks of culture (b).

Figure 5. Root response, (a) root initiation, b) root proliferation (after 20 days) and (c) root proliferation (after 30 days).

due to degradation and conjugation of cytokinin with sugar or amino acid to form biologically inactive complex (Tran and Trin, 1990).

For rooting response, *in vitro* raised shoots were transferred to full strength basal media. Among the different combinations and concentrations of plant growth regulators namely, IAA, IBA, NAA and 2-4 D used, MS + IBA (0.5 mg/l) was found to be most effective for rooting with 77.77% root induction factor, root length 6.0 cm and number of roots 28 in 12 days (Table 5 and Figure 5).

Figure 6. Hardening of plant material in plastic pot.

Figure 7. Fully developed plant after one month in transgenic glass house.

This result coincides with other observations where IBA was found to be most suitable auxin for rooting in many plant species (Parveen and Shahzad, 2010; Raghu et al., 2006; Shahzad et al., 2007). IBA was also found to be efficient for rooting in rhizome and tuber segment of high altitudinal Himalayan plant *Aconitum atrox* (Rawat et al., 1992), *Podophyllum hexandrum* (Nadeem et al., 2000) and *Cedrus deodara* (Nandi et al., 2002). It may be due to its decelerate interchange and degradation which assist its localization near the site of application and thus find its ability in root induction (Nickell and Kirk- Othmer, 1982). For lucrative formation of *in vitro* raised plants after transplantation, they were covered with polythene bags during the initial weeks to sustain clamminess for their survival (Thomas and Prasad, 2005) as they are highly susceptible to transplantation shock due to delicate root system, moderate amount of epicuticular wax and condensed stomata (Hazarika, 2003). Thus, the afore-mentioned standardized protocol is proficient for mass propagation of this endemic species from minimal fraction of plant material and thus restrains it from the verge of extermination.

Table 1. Effect of different phytohormones for callus induction from leaf explant.

Hormone (mg L^{-1})	Days to callus initiation	% callus induction factor ± SE	Fresh weight (g)	Colour	Growth
MS (control)	-	-	-	-	**
2,4-D (0.5)	29	50 ± 0.11	0.108	Cream	+
2,4-D (1.0)	22	58.33 ± 0.33	0.124	Green	+
NAA (0.5)	23	25 ± 0.05	0.104	Green	+
NAA (1.0)	17	41.66 ± 0.16	0.613	Green	+++
IBA (0.5)	25	33.33 ± 0.17	0. 110	Green	+
IBA (1.0)	22	66.66 ± 0.33	0. 146	Green	+
TDZ (0.5)	-	-	-	-	**
TDZ (1.0)	21	41.66 ± 0.24	0. 292	Green	+
BAP (0.5) + NAA (0.3)	13	75 ± 0.17	1.326	Green	++++
BAP (0.3) + NAA (0.5)	14	58.33 ± 0.33	0.892	Green	+++
BAP (0.5) + NAA (1.0)	21	66.66 ± 0.33	0.672	Green	++++
BAP (1.0) + NAA (0.5)	15	50 ± 0.00	0.809	Green	++++
BAP (1.0) + NAA (1.0)	22	75 ± 0.57	0.624	Green	+++
BAP (2.0) + NAA (1.5)	19	41.66 ± 0.33	0.326	Green	+++
BAP (1.5) + NAA (2.0)	20	33.33 ± 0.16	0.421	Green	+++
BAP (0.3) + 2,4-D (0.5)	26	50 ± 0.11	0.223	Green	++
BAP (1.0) + 2,4-D (0.5)	23	66.66 ± 0.33	0.412	Green	+++
BAP (1.5) + 2,4-D (2.0)	25	16.66 ± 0.24	0.398	Green	++
TDZ (0.5) + NAA (0.3)	28	41.66 ± 0.17	0.213	Green	+
TDZ (1.0) + NAA (1.5)	23	50 ± 0.19	0.219	Green	+
TDZ (2.0) + NAA (1.0)	32	58.33 ± 0.33	0.314	Green	+

**, no response; +, poor; ++, fair; +++, average; ++++, good. SEM: 0.256, CD at 5% = 0.730.

Table 2. Effect of different phytohormones for callus induction from stem explant.

Hormone (mg L^{-1})	Days to callus initiation	% callus induction factor ± SE	Fresh weight (g)	Colour	Growth
MS (control)	-	-	-	-	**
2,4-D (0.5)	27	66.66 ± 0.33	0.128	Cream	+
2,4-D (1.0)	23	33.33 ± 0.33	0.212	Green	+
NAA (0.5)	20	41.66 ± 0.33	0.214	Green	+
NAA (1.0)	18	58.33 ± 0.33	0.413	Green	+++
IBA (0.5)	23	50 ± 0.11	0. 210	Green	+
IBA (1.0)	23	50 ± 0.11	0. 246	Green	+
TDZ (0.5)	21	33.33 ± 0.16	0. 102	Green	+
TDZ (1.0)	24	50 ± 0.17	0. 192	Green	+
BAP (0.5) + NAA (0.3)	15	83.33 ± 0.05	0.996	Creamish green	++++
BAP (0.3) + NAA (0.5)	15	75 ± 0.17	0.798	Light green	++++
BAP (0.5) + NAA (1.0)	22	75 ± 0.05	0.762	Green	++++
BAP (1.0) + NAA (0.5)	17	50 ± 0.11	0.609	Creamish green	++++
BAP (1.0) + NAA (1.0)	20`	50 ± 0.17	0. 624	Light green	+++
BAP (2.0) + NAA (1.5)	20	41.66 ± 0.33	0. 562	Creamish green	+++
BAP (1.5) + NAA (2.0)	18	58.33 ± 0.33	0.596	Light green	+++
BAP (0.3) + 2,4-D (0.5)	25	66.66 ± 0.33	0.623	Green	+++
BAP (1.0) + 2,4-D (0.5)	23	75 ± 0.17	0.662	Green	+++
BAP (1.5) + 2,4-D (2.0)	25	66.66 ± 0.33	0.598	Green	++
TDZ (0.5) + NAA (0.3)	27	50 ± 0.23	0.393	Green	+

Table 2. Contd

TDZ (1.0) + NAA (1.5)	22	58.33 ± 0.33	0.529	Green	+
TDZ (2.0) + NAA (1.0)	32	25 ± 0.11	0.274	Green	+

**-, No response; +, poor; ++, fair; +++, average; ++++, good. SEM: 0.239, CD at 5% = 0.682.

Table 3. Effect of different combinations of BAP and kinetin on shoot induction from leaf callus.

Hormone (mg L^{-1})	Days to shoot initiation	Shoot induction factor ± SE	Number of shoots/explant	Average shoot length (cm)
BA (0.0) + KN (0.0)	-	-	-	-
BA (0.0) + KN (0.25)	-	-	-	-
BA (0.0) + KN (0.5)	18	33.33 ± 0.05	2	3.2
BA (0.0) + KN (0.75)	-	-	-	-
BA (0.0) + KN (1.0)	21	66.66 ± 0.11	4	3.6
BA (0.25) + KN (0.0)	21	55.55 ± 0.16	5	4.6
BA (0.75) + KN (0.0)	18	33.33 ± 0.11	2	1.5
BA (0.5) + KN (0.0)	16	88.88 ± 0.16	6	5.6
BA (1.0) + KN (0.0)	18	66.66 ± 0.11	5	5.7
BA (0.25) + KN (0.5)	23	22.22 ± 0.13	4	2.9
BA (0.5) + KN (0.25)	21	66.66 ± 0.11	3	4.0
BA (0.75) + KN (0.25)	18	44.44 ± 0.16	2	3.8
BA (0.25) + KN (0.75)	21	22.22 ± 0.13	3	4.0
BA (0.5) + KN (0.5)	19	33.33 ± 0.11	4	2.7
BA (0.25) + KN (0.25)	22	11.11 ± 0.33	4	3.0
BA (0.75) + KN (0.75)	23	33.33 ± 0.05	3	4.0
BA (1.0) + KN (1.0)	22	55.55 ± 0.17	5	3.1

SEM: 0.114, CD at 5% = 0.330.

Table 4. Effect of different combinations of BAP and kinetin on shoot induction from stem callus.

Hormone (mg L^{-1})	Days to shoot initiation	Shoot induction factor ± SE	Number of shoots/explant	Average shoot length (cm)
BA (0.0) + KN (0.0)	-	-	-	-
BA (0.0) + KN (0.25)	-	-	-	-
BA (0.0) + KN (0.5)	20	55.55 ± 0.16	4	2.7
BA (0.0) + KN (0.75)	-	-	-	-
BA (0.0) + KN (1.0)	22	66.66 ± 0.11	3	3.8
BA (0.25) + KN (0.0)	20	33.33 ± 0.05	3	4.1
BA (0.75) + KN (0.0)	16	77.77 ± 0.33	8	4.6
BA (0.5) + KN (0.0)	19	66.66 ± 0.17	5	5.4
BA (1.0) + KN (0.0)	19	88.88 ± 0.33	4	3
BA (0.25) + KN (0.5)	21	33.33 ± 0.11	3	3.5
BA (0.5) + KN (0.25)	20	66.66 ± 0.05	5	4.8
BA (0.75) + KN (0.25)	18	33.33 ± 0.05	4	3.2
BA (0.25) + KN (0.75)	22	11.11 ± 0.03	3	3
BA (0.5) + KN (0.5)	17	33.33 ± 0.00	5	4
BA (0.25) + KN (0.25)	23	11.11 ± 0.03	4	3
BA (0.75) + KN (0.75)	25	22.22 ± 0.03	3	3.8
BA (1.0) + KN (1.0)	22	55.55 ± 0.16	4	4.2

SEM: 0.143, CD at 5% = 0.411.

Table 5. Effect of different phytohormones on root induction.

Hormone (mg L^{-1})	Days to root initiation	Root induction factor ± SE	Number of roots/explant	Average root length (cm)
MS (control)	-	-	-	-
IAA (0.5)	16	33.33 ± 0.02	19	1.6
IAA (1.0)	19	44.44 ± 0.06	16	1.4
IBA (0.5)	12	77.77 ± 0.01	28	6.0
IBA (1.0)	14	55.55 ± 0.06	23	5.8
NAA (0.5)	14	55.55 ± 0.06	24	5.7
NAA (1.0)	17	33.33 ± 0.02	21	5.6
2,4-D (0.5)	-	-	-	-
2,4-D (1.0)	22	11.11 ± 0.01	13	1.0
2,4-D (0.5) + NAA (1.0)	19	22.22 ± 0.009	15	2.0
2,4-D (0.5) + IBA (1.0)	17	44.44 ± 0.02	20	1.9
2,4-D (0.5) + IAA (1.0)	19	22.22 ± 0.009	22	2.1
IAA (0.5) + IBA (1.0)	15	44.44 ± 0.07	21	2.4
IAA (1.0) + IBA (0.5)	18	33.33 ± 0.02	18	2.9
IAA (1.0) + NAA (0.5)	19	55.55 ± 0.06	16	3.2
IAA (0.5) + NAA (1.0)	15	44.44 ± 0.06	16	2.9
IBA (1.0) + NAA (0.5)	15	55.55 ± 0.05	17	3.7
IBA (0.5) + NAA (1.0)	19	44.44 ± 0.06	12	3.9

SEM: 0.477, CD at 5% = 0.136.

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