Overcoming Early Shoot Senescence of *Colutea istria* Miller Propagated *In Vitro*

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Abstract: Seedlings of the leguminous shrub; *Colutea istria* Mill. were used as explants for the micropropagation of this vulnerable species. Cotyledonary nodes, stem node sections and shoot tips from the *in vitro* germinated seedlings were examined for micropropagation. The *in vitro* establishment of explants was attempted by using various concentrations of 6-benzylaminopurine (BA), Thidiazuron (TDZ) and N6-(2-isopentenyl) adenine (2iP) (0.5, 1, 2 mg/L each) in combination with NAA at 0.1 and 0.2 mg/L incorporated into Murashige and Skoog (MS) and Gamborg's (B5) media, in addition to the MS and B5 media without plant growth regulators (PGRs). The best results were obtained on MS medium supplemented with NAA and BA, in addition to PGRs free MS medium. And the best average number and length of shoots were obtained by using cotyledonary nodes as explants. For multiplication, the explants were cultured on MS medium containing BA at concentrations of 0.25, 0.5 and 1 mg/L either individually or in combination with 2iP at a concentration of 0.5 mg/L. The combination of BA and 2iP is recommended for multiplying the established shoots produced from colyledonary nodes and stem node sections due to the significantly higher average number of shoots/explant comparing to the media containing BA singly. However, BA is better for the multiplication of shoot tip explants. When axillary shoots were subcultured on the same medium, the shoots failed to multiply and began to senesce. The senescence progressed to the entire shoot, and growth ceased. Reducing the duration of the subculture to 3 weeks is necessary to prevent this problem. Explants rooted on MS medium containing 0.5 mg/L of both Indole-3-butyric acid (IBA) and NAA and plantlets with well developed shoots and roots transferred to soil and grew normally without loss of green colour and wilting.

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Key Words: *Colutea Istria,* Leguminosae, micropropagation, *in vitro* culture, seedlings, yellowing, senescence.

Abbreviations: AgNO3, Silver Nitrate; BA, 6-Benzyladenine; CaCl2, Calcium Chloride; IBA, Indole-3-Butyric Acid; 2iP, N6-(2-Isopentenyl) Adenine; MS, Murashige and Skoog; NAA, δ-Naphthalene Acetic Acid; NaOCl Sodium Hypochlorite; PGRs, Plant Growth Regulators; TDZ, Thidiazuron.

Introduction

The economical and ecological importance of leguminous forest trees necessitated the application of micropropagation technique for their clonal multiplication **(Pradhan *et al,* 1998)**, furthermore, the regeneration rate of leguminous trees in natural habitats is low **(Dewan *et al,* 1992)**. Also, as stated by **Nanda and Rout (2003)**, due to poor germination and death of young seedlings under natural conditions, propagation through seeds, as with most leguminous trees is unreliable. Tissue culture techniques have been used for rapid clonal multiplication of selected genotypes of a number of forest trees including some woody legumes **(Dhawan, 1989 and Trigiano *et al,* 1993)**. During the past few years, a number of woody legumes have been successfully propagated *in vitro* using juvenile as well as mature plant parts **(Pradhan *et al,* 1998)**.

Colutea istria Mill. is a very rare species in Egypt and confined to Sinai which seems to be the westernmost limit of the species distribution. Its Arabic name is Yasser. The genus *Colutea* is belonging to the family Fabaceae (Leguminosae). It is a nano-phanerophyte which grows on crevices of smooth-faced rocky outcrops. Flowering and fruiting of this species is from February to April. *Colutea istria* is a vulnerable shrub. Its vulnerability can be related to its small population size combined with cutting by the natives for the preparation of ropes from the bark fibers. Older branches are used as firewood grazing by domestic livestock leading to the gradual decline of the species. Two populations of *Colutea istria* in Gebel El Rabba (Sant Catherine, South Sinai) and Gebel El Maghara (North Sinai) are being wildlife sanctuaries. Cultivation in botanical gardens as an ornamental is another useful method to protect the species from extinction. The plant is of horticultural value, because of its habit and decorative flowers **(El-Hadidi *et al,* 1991)**. The bark and fibers are used for the preparation of strong ropes **(Danin, 1983)**. It is a highly palatable, has a high nutritive value and is used as a fodder plant.

The seed oils of *Colutea* contained linoleic, oleic and linolenic acids as their major components. Palmitic and stearic acids were the major saturated fatty acids in the seed oils **(Bagci, 2004)**. Sixteen flavonoidal compounds from *Colutea istria* were isolated and identified as: quercetin-3-glucoside; quercetin-3-gentiobioside; quercetin-3,7-diglucoside; isorhamnetin-3-gentiobioside; isorhamnetin-3-rutinoside; kaempferide-3,7-diglucoside; 2',4,4'trihydroxychalcone; 2',4,4',6'tetrahydroxychalcone; formononetin; daidzein; calycosin; cladrin; rhamnocitrin-3-neohesperidoside; rhamnocitrin-3-glucoside; rhamnocitrin-3-galactoside and rhamnocitrin aglycone **(Shabana *et al,* 2005)**. Two new isoflavonoids have been isolated from the aerial parts of *Colutea istria* of Egyptian origin and identified as (3)-7-hydroxy-3',4'-dimethoxyisoflavan-2',5'-quinone and 6,3'-dihydroxy-7,4'-dimethoxyisoflavone **(Radwan, 2008)**.

Hegazi (2005) studied the response of *Colutea istria* Mill. to *in vitro* propagation from nodal segments and shoot tip explants from the mature plants. Explants were cultured on Murashige and Skoog (MS) nutrient medium **(Murashige and Skoog, 1962)** supplemented with different concentrations of 6-benzyladenine (BA) alone or in combination with 0.1 mg/L δ-Naphthalene acetic acid (NAA). In the establishment stage of the nodal segments; MS nutrient medium supplemented with 3 mg/L BA attained the highest average number of axillary shoots/explants. However, MS nutrient medium with 3 mg/L BA and 0.1 mg/L gave the highest value of the average length

of axillary shoots. MS medium supplemented with 4 mg/L BA with or without 0.1 mg/L NAA were the best media for the establishment of shoot tip explants. Nodal segments showed better response to the *in vitro* establishment than shoot tips. The regenerated shoots from both types of explants were multiplied on the best establishment medium. Shoots showed a reduced ability to elongate and became senescent, then abscised. To overcome this problem, different treatments were applied as the elimination of BA from the medium, doubling the amount of Calcium Cloride (CaCl2) in the medium, using the double phase medium, addition of Silver nitrate (AgNO3) to the medium, at different concentrations (1, 2 and 5 mg/L) and reducing the sucrose concentration to 20 g/L. All these treatments did not prevent the yellowing and necrosis of the shoots.

The aim of this study is to develop a micropropagation protocol for *Colutea istria* Miller using seedling explant materials because juvenile explants generally perform better in tissue culture than do mature explants, and to overcome early shoot senescence and obtain complete plantlets of this important endangered plant, to conserve and protect it from extinction. Since, there is no information on the micropropagation of Genus *Colutea* except the previous review.

Materials and Methods

Plant material, media and culture conditions

Seeds of *Colutea istria* Mill. were collected from their natural habitat; Gebel El-Maghara in Wadi El-Arousia. They were washed under running tap water and surface sterilized by immersion into 50% concentration of commercial bleach (active ingredient, 5.25% NaOCl) for 20 min, and then rinsed five times with sterile distilled water.

Seeds were individually planted on MS medium free from plant growth regulators (PGRs). The percentage of survival and seed germination were recorded after three weeks of culture. Cotyledonary nodes, stem node sections and shoot tips from the seedlings were examined for *in vitro* propagation. The *in vitro* establishment of explants was attempted by using various concentrations of BA, TDZ and 2iP (0.5, 1, 2 mg/L each) in combination with NAA at 0.1 or 0.2 mg/L incorporated into MS and Gamborg's (B5) media, in addition to the control media without PGRs, to select the optimum hormonal combination and concentration for shoot development.

All media were supplemented with 100 mg/L myo-inositol, 30 g/L sucrose and gelled with 2.5 g/L phytagel. The pH was set at 5.7-5.8, adjusted with 0.1N NaOH and HCl. Fifteen ml volumes of the media were dispensed into 25×150 mm glass culture tubes for explant establishment and rooting, or 45-50 ml volumes into large jars for seed germination and proliferation of shoots. All containers were closed with autoclavable polypropylene caps and autoclaved for 15 minutes at 121˚C under a pressure of 1.1 Kg/cm². The cultures were incubated at a temperature of 26±2˚C and were exposed to a 16-h photoperiod supplied by a bank of cool-white florescent tubes of 2 K lux light intensity.

For multiplication, the explants were cultured on MS medium containing BA at concentrations of 0.25, 0.5 and 1 mg/L and 2iP at a concentration of 0.5 mg/L – either individually or in combination, to obtain stock materials to be used for the following stages. MS medium without PGRs was also tested.

Thirty replicates were used for each treatment, and each experiment was repeated thrice during 2008-2010. The percentage of explants regenerating axillary growth, the average number of axillary shoots/explant and the average length of axillary shoots (cm) were recorded after 3 weeks of culture.

For root induction, the *in vitro* developed shoots were planted on MS medium supplemented with various concentrations of IBA and NAA (0.1, 0.2, 0.5, 1, 2 mg/L) individually and in combination, as following; 0.1 mg/L IBA+0.1 mg/L NAA, 1 mg/L IBA+0.2 mg/L NAA, 1 mg/L IBA+1 mg/L NAA and IBA at concentrations of 0.5, 1 and 2 in combination with 0.5 mg/L NAA. Explants with well developed roots were placed in pots containing garden soil and peat moss in a ratio of 1:1 and covered with plastic bags to maintain high humidity. The pots were irrigated regularly.

Treatments for overcoming shoot senescence

Treatments tested to overcome shoot senescence and necrosis of the plantlets were 1. Addition of L-glutamine at 100 mg/L – either individually or in combination with thiamine-HCl at 40 mg/L. 2. Reduction of the duration of the subculture from 4-6 weeks as recorded by **Hegazi (2005)** to 3 weeks. 3. Reducing the concentration of BA and addition of 2iP to the medium. 4. Transferring the shoots into growth regulators free medium after each subculture

Analysis of data

Analysis of variance (ANOVA) and Duncan's multiple range test were performed to analyze the obtained data. The differences among means for all treatments were tested for significance at 5% level. Means followed by the same letter are not significantly different at $P \leq 0.05$.

Results and discussion

Seeds germination and establishment stage

The seeds were germinated on MS medium free from PGRs and germination percentage reached 44.2% after 3 weeks of culture (Figure 1A). An experiment was conducted testing the establishment of the three types of explants developed from the germinated seedlings; cotyledonary nodes, stem node sections and shoot tips, cultured on MS and B5 media supplemented with NAA at 0.1 or 0.2 mg/L in addition to three different cytokinins; BA, TDZ and 2iP, at concentrations of 0.5, 1 and 2 mg/L as shown in table 1 (Plate 1B).

Concerning the establishment stage (Figure 1B), table 1 indicates that the use of MS nutrient medium was more effective than B5 medium with respect to the average number and length of axillay shoots for all types of explants. This finding is agreed with **Vengadesan *et al* (2002)** who reported that MS medium was preferred in the micropropagation of many leguminous trees. For shoot tips there was insignificant difference in the average shoot length between MS medium supplemented with 0.1 mg/L NAA+2 mg/L BA and B5 medium supplemented with 0.2 mg/L NAA+0.5 or 1 mg/L BA. And the highest value of the average length of axillary shoots was obtained on MS medium containing 0.1 mg/L NAA+0.5 or 1 mg/L BA. Shoot tips gave the highest percentage of explants regenerated axillary growth (100%) on most of the tested media if compared with the other types of explants. The suitability of shoot tip explants for regeneration and its sensitivity to various hormones is due to the activity of meristematic cells, which are actively dividing and are known to have dense cytoplasm with much more uniform and homogenous composition **(Mathur *et al*, 2002a)**. Also, cotyledonary nodes gave 100% of explants regenerated axillary growth on B5 medium without PGRs and B5 medium+0.2 mg/L NAA+0.5 and 2 mg/L 2iP. However, stem node sections gave the highest percentage of explants regenerated growth (83%) on MS medium supplemented with 0.1 mg/L NAA+1 mg/L BA.

It is noticed that the best medium with respect to the average number of axillary shoots derived from cotyledonary nodes, was MS medium containing 0.1 mg/L NAA+0.5 mg/L BA. However, MS medium without PGRs gave the best average length of axillary shoots (3.6). **Abd Alhady *et al* (2010)** have also recorded similar results on

MS control medium in the establishment of *Periploca angustifolia*. Stem node sections gave the highest number of axillary shoots/explant on MS medium supplemented with 0.1 mg/L NAA+2 mg/L BA, and it significantly decreased with the decrease of BA concentration. The highest average length of axillary shoots was obtained on MS medium supplemented with 0.1 mg/L NAA+0.5 mg/L BA. Shoot tips developed the maximum number of axillary shoots/explant on MS medium containing 0.1 mg/L NAA+0.5 and 1 mg/L BA which decreased significantly with the increase of BA concentration.

Comparing the three tested cytokinins added to B5 medium; differences were observed in the data obtained. It could be noticed that TDZ at 0.5 mg/L followed by 2iP at 2 mg/L gave the highest average number of axillary shoots/explants, and 2iP at concentrations of 1 and 2 mg/L proved to be the best with respect to the average length of axillary shoots developed from cotyledonary nodes. Also, **Scholten (1998)** found that Lilac tree (*Syringa vulgaris*) needed 2iP for elongation and it is used to induce bud growth in many *Acacia* species **(Vengadesan *et al*, 2002)**. Concerning stem node sections; BA and TDZ at concentrations of 1 and 2 mg/L in addition to all the tested concentrations of 2iP (0.5, 1 and 2 mg/L) were insignificantly different in the average number of axillary shoots/explant. Also, the average length of axillary shoots was insignificantly different on all B5 tested media. With respect to shoot tip explants, there were insignificant differences between the three tested cytokinins in the average number of axillary shoots/explants. Although, average length of axillary shoots was significantly the highest on B5 medium containing 0.5, 1 mg/L BA and 2 mg/L TDZ. The B5 control medium without PGRs and B5 medium containing 0.2 mg/L NAA are not recommended for the *in vitro* establishment of *C. istria* plant due to the least or no response of the explants.

In conclusion, the best medium for establishment of all tested explants was MS medium supplemented with NAA and BA, in addition to PGRs free MS medium. This result confirmed by **Al-Wasel (2000)** who obtained multiple shoots from shoot tips of seedlings of *Acacia seyal* when BA and NAA were used together. He found that no clear trend was observed by TDZ. BA in combination with an auxin was also found to be essential for multiple shoot induction in some other leguminous trees as many *Acacia* species **(Shekhawat *et al*, 1993; Bhaskar and Subhash, 1996** and **Vengadesan *et al*, 2002)**. In addition, **Abd Alhady *et al* (2010)** found that the combination of the two cytokinins (BA and 2iP) was more ideal for shoot multiplication than 2iP singly. The best average number and length of axillary shoots were obtained by using cotyledonary nodes as explants. It may contribute to the role of cotyledonary nodes in shoot production from seedling explants as they supply endogenous growth regulators to the cultures as reviewed by **Audiehya (1999)**. Also, cotyledonary nodes were used successfully for the micropropagation of *Acacia mangium* **(Vengadesan *et al*, 2002)**.

Multiplication stage

With respect to the multiplication stage (Figure 1C), data shown in table 2 indicates that cotyledonary nodes gave the maximum average number of axillary shoots/explant on MS medium supplemented with 0.5 mg/L of both BA and 2iP followed by 0.25 mg/L BA which is the best medium in the average length of axillary shoots. The average length of axillary shoots decreased with the increase in BA concentration. Similar observation was reported by **Abd Alhady *et al* (2010)**. There were insignificant differences in the average length of axillary shoots between the MS medium containing 0.5 and 1 mg/L BA and that containing 0.25 and 0.5 mg/L BA+0.5 mg/L 2iP. Concerning stem node sections, MS medium containing 0.25 mg/L BA+0.5 mg/L 2iP induced the highest average number of axillary shoots/explants, followed by MS medium without PGRs and MS+0.5 mg/L of both BA and 2iP which is insignificantly different from the same concentration of BA (0.5 mg/L) without 2iP. Both 0.25 and 0.5 mg/L BA were optimum for the production of the highest average length of axillary shoots, followed by 0.5 mg/L 2iP. Shoot tip explants gave the highest number of axillary shoots/explant on MS medium with 1 mg/L BA and decreased with the decrease of BA concentration or addition of 2iP to the medium. With respect to the average length of axillary shoots, it reached the highest value on MS medium containing 0.25 mg/L BA comparing to the other tested media. BA as a cytokinin proved to be effective *in vitro* with many woody species **(Murashige, 1974; Sharma *et al*, 1981 and Bennett and Davies, 1986)**. Also, it is the most frequently used compound in enhancing the production of proliferated shoots **(Thomas and Blackesly, 1987)**.

In conclusion, the combination of BA and 2iP is recommended for multiplying the established shoots produced from colyledonary nodes and stem node sections due to the significantly higher average number of axillary shoots/explant comparing to the media containing BA singly. However, BA is better for the multiplication of shoot tip explants. It was reported that an excess of synthetic cytokinin like BA is the most effective for the *in vitro* microporopagation of *Acacia mangium* **(Darus, 1993)**.

When axillary shoots were subcultured on the same medium, the shoots failed to multiply and leaves began to senesce. At the same time the leaf senescence progressed to the entire shoot and growth ceased. A similar observation was reported by **Hegazi (2005)** during the multiplication of of *Colutea istria* shoot tips and nodal segments from mature plants, and in other leguminous plants as *Leucaena leucocephala* **(Dhawan and Bhojwani, 1985)**, *Dalbergia latifolia* **(Swamy *et al*, 1992)** and *Dalbergia sissoo* **(Gulati and Jaiwal, 1996)**. For this reason it was important to apply a series of treatments to overcome such a problem.

Treatments for overcoming shoot senescence

Addition of L-glutamine at 100 mg/L either singly or in combination with Thiamine-HCl at 40 mg/L failed to inhibit yellowing and caused complete death of the explants. This result is in contrary with that of **Ranga Rao and Prasad (1991)** who mentioned that the amino acid L-glutamine increases shoot bud regeneration, and **Vengadesan *et al* (2002)** who found that it is ideal for shoot bud induction in *Acacia catechu* and *A. nilotica*. Also, **Mathur *et al* (2002b)** reported that incorporation of additives as glutamine and thiamine HCl was found to be most effective in shoot elongation as well as accelerating multiple shoot proliferation. Glutamine proved to be most effective to stop leaf-fall in multiple shoots, and thiamine is known to be one of the most important vitamin additives. Although green plant parts normally synthesize thiamine, additional amounts to the culture medium appeared to stimulate explants growth and may enhance root growth in the rooting stage.

The reduction of the duration of the subculture from 4-6 weeks as recorded by **Hegazi (2005)** to 3 weeks was very effective in the deterioration of the yellowing of the explants. This observation is supported by **Vengadesan *et al* (2002)** who found that in genus *Acacia*, frequent subculture of explants in constant intervals (25-30 days) make significant improvement in enhancing the number of multiple shoots. Repeated sub-culturing caused activation and conditioning of meristems. Two continuous subcultures excluding the initial culture favoured maximum shoot multiplication in *A. mangium*, *A. nilotica*, *A. sinulata*. Transferring the cultures to fresh

medium after three weeks found to be essential to prevent culture deterioration and sustained shoot growth.

Also, reducing the concentration of BA and addition of 2iP to the medium, gave promising results in the reduction of leaf senescence, since **Hegazi (2005)** used BA at high concentrations reached to 4 mg/L which caused shoot senescence in the multiplication stage. In this experiment the concentrations of BA reduced (0.25, 0.5 and 1 mg/L) and the percentage of explants regenerated growth and survived from yellowing ranged between 40% and 100%. Growth regeneration decreased with the increase of cytokinins concentration or the elimination of BA. It is concluded that BA is very important for the multiplication of shoots but if added with high concentrations it causes yellowing and necrosis of the explants. Also, the regular transferring of the shoots into fresh PGRs free medium after each subculture gave promising results in protecting the shoots. In this respect, **Al-Wasel (2000)** found that shoots of *Acacia seyal* needed to be subcultured into PGRs-free medium to allow and maintain shoot survival and elongation. These results are in harmony with that recorded by **Galiana *et al* (1991)** and **Darus (1993)** who mentioned that high concentrations of BA were observed to stimulate greatly the potential for axillary shoot formation during the first subcultures, but resulted quickly in a noticeable and often irreversible organogenic culture decline. It may rapidly become phytotoxic after a small number of subcultures, as noticed in other species. Also, **Rosu *et al* (1995)** reported that BA appears to have a positive effect only in the initial stage of culture establishment. This effect did not persist because of the leaf senescence which was presumably induced by ethylene. BA has been shown to stimulate ethylene biosynthesis *in vitro* **(Escalettes** and **Dosba, 1993)**.

Rooting and acclimatization

Only two media gave response in root induction; MS medium supplemented with 0.1 mg/L NAA which induced the rooting of 10% of axillary shoots and MS medium containing 0.5 mg/L of both IBA and NAA, on which 40% of explants developed roots (Figure 1D). These results is attributed to the presence of a number of explants that became senescent and caused the yellowing which didn't permit the auxins tested for promoting root formation. Fifty percent of the plantlets with well developed shoots and roots transferred to soil and grew normally without loss of green colour and wilting over 4 weeks observation period (Figure 1E).

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Table 1. Establishment of different seedling's explants of *Colutea istria* cultured on MS and B5 nutrient media supplemented with different growth regulators.

Nutrient medium	PGR concentration (mg/L)				Cotyledonary nodes			Nodal segments			Shoot tips		
	NAA	BA	TDZ	2iP	% of explants regenerated growth	Average number of axillary shoots/ explant	Average length of axillary shoots (cm)	% of explants regenerated growth	Average number of axillary shoots/ explant	Average length of axillary shoots (cm)	% of explants regenerated growth	Average number of axillary shoots/ explant	Average length of axillary shoots (cm)
MS	-	-	-	-	66.66	2.4 c	3.60 a	33.33	1.6 b	2.70 b	100.0	2.2 c	1.04 bc
	0.1	0.5	-	-	66.66	6.0 a	2.00 b	66.66	1.6 b	3.46 a	66.66	5.4 a	2.10 a
	0.1	1	-	-	66.66	3.4 b	2.00 b	83.33	2.0 b	1.82 c	66.66	5.2 a	2.52 a
	0.1	2	-	-	66.66	3.0 b	1.90 b	66.66	3.0 a	1.34 c	66.66	3.0 b	1.20 b
B5	-	-	-	-	100.0	1.0 de	1.52 bc	00.00	0.0	0.00	100.0	1.0 d	0.50 cd
	0.2	-	-	-	00.00	0.0	0.00	00.00	0.0	0.00	100.0	1.0 d	0.58 cd
	0.2	0.5	-	-	66.66	1.0 de	0.82 def	00.00	0.0	0.00	100.0	1.4 d	1.48 b
	0.2	1	-	-	66.66	1.0 de	0.50 efg	20.00	1.0 c	0.24 d	50.00	1.0 d	1.20 b
	0.2	2	-	-	66.66	0.6 e	0.22 g	80.00	1.0 c	0.56 d	100.0	1.0 d	0.38 d
	0.2	-	0.5	-	33.33	2.0 c	1.00 cde	00.00	0.0	0.00	100.0	1.0 d	1.00 bc
	0.2	-	1	-	66.66	1.0 de	0.50 efg	40.00	1.0 c	0.24 d	100.0	1.0 d	1.00 bc
	0.2	-	2	-	33.33	1.0 de	0.48 efg	20.00	1.0 c	0.22 d	100.0	1.0 d	1.16 b
	0.2	-	-	0.5	100.0	1.0 de	0.38 fg	40.00	1.0 c	0.20 d	66.66	1.0 d	0.50 cd
	0.2	-	-	1	66.66	1.0 de	1.06 cd	20.00	1.0 c	0.22 d	66.66	1.0 d	0.92 bcd
	0.2	-	-	2	100.0	1.4 d	1.28 cd	80.00	1.0 c	0.22 d	100.0	1.0 d	0.90 bcd

Table 2. Multiplication of different seedling's explants of *Colutea istria* cultured on MS nutrient medium supplemented with BA and 2iP.

Cytokinin concentration (mg/L)		Cotyledonary nodes			Nodal segments			Shoot tips		
BA	2iP	% of explants regenerated growth	Average number of axillary shoots/ explant	Average length of axillary shoots (cm)	% of explants regenerated growth	Average number of axillary shoots/ explant	Average length of axillary shoots (cm)	% of explants regenerated growth	Average number of axillary shoots/ explant	Average length of axillary shoots (cm)
-	-	66.66	1.8 abc	2.20 b	50.00	2.0 b	1.1 bc	62.50	1.6 ab	1.8 b
0.25	-	100.0	2.2 ab	3.60 a	50.00	1.0 d	1.0 bc	100.0	1.6 ab	2.62 a
0.50	-	100.0	1.0 cd	2.40 ab	100.0	2.0 b	2.6 a	100.0	1.8 ab	1.5 b
1.00	-	100.0	1.0 cd	2.70 ab	100.0	1.0 d	0.5 cd	60.00	2.3 a	1.7 b
-	**0.5**	95.00	1.6 abcd	1.90 b	41.33	1.4 c	1.7 b	96.66	1.4 ab	1.5 b
0.25	**0.5**	100.0	1.4 bcd	2.80 ab	100.0	3.0 a	2.6 a	100.0	1.0 b	0.5 c
0.50	**0.5**	100.0	2.6 a	2.40 ab	100.0	2.0 b	1.0 bc	100.0	1.4 ab	1 bc
1.00	**0.5**	40.00	0.6 d	0.16 c	00.00	0.0	0.0	100.0	1.4 ab	1.8 b

A

B

C

D

E

Figure 1. Different stages of micropropagation of *Colutea istria*.
A. *In vitro* germinated seedlings on MS medium free from PGRs. B. Establishment of cotyledonary nodes and shoot tip explants. C. Multiplication of axillary shoots on MS medium supplemented with BA and 2iP. D. Rooted plantlets on MS medium+0.5 mg/L NAA+0.5 mg/L IBA. E. Complete plantlets transfered to soil.