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Indirect Organogenesis from *in vitro* Derived Leaf and Internodes of *Coccinia cordifolia* (L.) Cogn. - An Important Medicinal Climber

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Abstract

An efficient *in vitro* regeneration protocol was developed via callus from the explants of leaf and internode of *Coccinia cordifolia* (L.) Cogn., an important medicinal climber. Among the different combinations of auxins and cytokines used, BAP with NAA were found highly effective for induction of callus from *in vitro* derived leaf disc and internode explants. Maximum callus formation rates were recorded on MS with 1.0 mg/l BAP and 0.1 mg/l NAA for both leaf disc and internode explants. The best organogenesis via callus was obtained on MS with 2.0 mg/l BAP and 0.5 mg/l NAA. The highest numbers of shoots regenerated per culture were 6.5 ± 0.10 and 5.6 ± 0.55 for leaf disc and internode explants respectively and leaf discs was found to be better for indirect organogenesis. For root induction, half strength of MS medium supplemented with 0.1 mg/l IBA was found to be the most effective. The mean number of roots regenerated per shoot was 6.8 ± 0.10 with an average root length of 2.8 ± 0.20 cm. *In vitro* derived plantlets were acclimatized successfully. This protocol for *in vitro* regeneration can be utilized for the improvement of this climber through biotechnological methods.

Introduction

Coccinia cordifolia (L.) Cogn. (Synonym: *Coccinia indica*, *Coccinia grandis*) is a slender dioecious perennial climber with tuberous roots; belongs to the family Cucurbitaceae and naturalized in jungles and on hedges all over Bangladesh. This plant is commonly known as Ivy gourd. This plant contains proteins, carbohydrates, vitamins, sterols, β-sitosterol, phenolic compounds, triterpenoid, β-amyrine, lupeol, alkaloids, glycoside (Cucurbitacin B), saponin and carotenoides. Saponin and flavonoids are found to be responsible for antidiabetic activity (Kashem and Rahman 2018).

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The whole plant extract of *C. grandis*, showed a strong free radical scavenging activity almost same as that of Ginseng (Gradist and Purintrapiban 2009). Leaf extract of Ivy gourd is effective against malarial parasites. The crude methanolic extract of Ivy gourd showed the highest larvicidal activity among the cucurbitaceous plants tested (Rahuman and Venkatesan 2008). In the global market, it is used as a principal ingredient in several products. The oral administration of *C. indica* leaves extract decreased the concentration of blood glucose, lipid and fatty acids. Fruit is applied to swelling and taken orally for disorder of blood, cure anaemia, dried root powder is cathartic, ash of root is applied for skin diseases and useful in bronchitis (Borah et al. 2019). Antimicrobial activities of *C. grandis* leaf and fruit extracts against several bacterial and fungal strains have also been reported (Farrukh et al. 2008). In cucurbits, normally the seed setting and seed germination is low due to the presence of a thin nuclear membrane lending impermeability to water and gases and making them dormant for many years. Problems associated with its cultivation include the shortage of seedling material from cuttings of mature stems and unrestricted exploitation by the pharmaceutical industries may lead to depletion of this plant resource (Thiripurasundari et al. 2012). Besides, such conventional propagation are season dependent, and most of the propagation can be achieved only during the monsoon period (Ghanthikumar et al. 2013).

Hence, *in vitro* techniques is a great alternative that can be employed to overcome these problems largely. Moreover, this has become an important aspect of plant science research including rapid propagation and conservation of traditional medicinal flora (Anisuzzaman et al. 2008a, Deokate and Khadabadi 2011). It may be pointed out here that organogenesis can be achieved indirectly through callus formation prior to the adventitious shoot and root development as well as directly where adventitious shoots or roots induce directly from various explants like leaf, petiole, internode, cotyledon, hypocotyl, etc. (Brown and Thorpe 1986). Organogenesis also help to development of varieties with new characteristics through somaclonal variation or genetic transformation (Pal et al. 2007, Anisuzzaman et al. 2008b). *In vitro* micropropagation can also pave the wave for isolating pharmacologically valuable phytochemicals from calli in a sustainable manner (Narayan 2016).

Few reports are available on *in vitro* propagation of *Coccinia* include direct shoot regeneration from hypocotyls explants (Gulati 1988), shoot tip and nodal segments (Sarkar et al. 2021, Kashem and Rahman 2018, Patel and Ishnava 2015, Sarker et al. 2009), direct and indirect regeneration from internodal segments (Borah et al. 2019, Josekutty et al. 1993). However, there are very few reports on indirect organogenesis using leaves and internodes. Hence, the present study was undertaken to develop an efficient and reproducible shoot initiation method through indirect organogenesis from leaf and internode to facilitate future plant genetic transformation experiments as well as in strengthening the conservation of this valuable plant also for the utilization of the plant resource for medicinal purposes when required.

Materials and Methods

Fresh, healthy explants of *Coccinia cordifolia* was collected from the northern side of the third science building, University of Rajshahi, Bangladesh and immature nodal segments were inoculated for axillary shoot regeneration. Then from *in vitro* grown leaf and internodes of 0.5-1.0 cm were excised for indirect organogenesis.

Stock solutions of micro and minor elements, vitamins, plant growth regulators were prepared in a conical flask with required quantities and stored at 4°C refrigerator. An adequate amount of all the nutrients, vitamins, growth regulators were poured into a beaker to prepare 1000 ml of MS medium. Additional distilled water was added to mark up the volume of 1L after dissolving 3% (w/v) sugar as a carbon source. The pH of the medium was then adjusted at 5.7 ± 0.1 with a digital pH meter by adding 0.1N NaOH or 0.1N HCl. After that, 0.75% (w/v) agar was added to solidify the medium and heated in a microwave for 5 min, and finally, the medium was sterilized by autoclaving for 20 min at 121°C temperature and 1 atmospheric pressure.

Field collected samples were sterilized with Savlon (ACI Ltd.) and tween 20 for 2-3 times with distilled water in a conical flask after rinsing under running tap water to eliminate surface dust and contaminants. Then these were treated with 0.1% $HgCl_2$ for 5-8 min before washing with sterile distilled water for 3-5 times. The surface sterilization of nodal segments was carried out in a laminar air-flow cabinet. Surface sterilized nodal segments were cultured on semisolid MS medium supplemented with various concentrations and combinations of BAP, Kn and NAA for axillary shoot development. Leaves and internodes collected from *In vitro* grown shoots were cultured on MS medium containing 1.0, 1.5 and 2.0 mg/l BAP individually or in combination with 0.1, 0.2 and 0.5 mg/l NAA, IBA, IAA, and 2, 4-D for callus induction and adventitious shoot regeneration. *In vitro* regenerated shoots were separated individually and were cultured in half-strength MS medium supplemented with 0.1 and 0.5 mg/l either of NAA, IBA, or IAA for induction of roots. All the *in vitro* cultures were carried out in a growth chamber having a light intensity of 2000-3000 lux with a 16 hrs photoperiod and the temperature was maintained at 25 ± 2°C. The rooted plantlets were then transplanted in a thumb pot containing sun sterilized sand and soil mixed with humus in the ratio of 1:2:1. The potted plants were then acclimatized regularly in an *ex vitro* environment after being kept for 15 days in the growth chamber. Data were recorded after 5 weeks of culture for 7 replications in each experiment with 3 technical replications. Microsoft Excel was used to calculate the average value and standard error of the obtained data.

Results and Discussion

In vitro grown leaf and internodes were used for indirect organogenesis via callus formation. The explants were cultured on MS semi solid media supplemented with different concentrations of either 2, 4-D alone or in combinations of BAP and with 2, 4-D. NAA, IBA with BAP in order to find out a suitable culture medium for development of suitable organogenic callus. Production of callus from leaf and nodal segments indicated

the induction of indirect organogenesis. Percentages of explants induced calli were recorded after 3-4 weeks of inoculation of the explants. The summarized results are presented at Table 1.

Table 1. Effects of various concentrations and combinations of auxins and a combination with cytokinin for callus induction from leaf disc and internode explants on MS medium.

Growth regulators (mg/l)	Types of explants	Response of explants (%) induced callus	Callus colour	Degree of callus formation
2,4-D 1.5	LD	70	FB	++
	IN	60	FB	++
BAP 1.0 + NAA 0.1	LD	90	FB	+++
	IN	80	FB	+++
BAP 1.0 + IBA 0.1	LD	80	FB	+++
	IN	65	FB	+++
2, 4-D 1.5 + BAP 0.2	LD	75	FG	+++
	IN	65	FG	+++
2, 4-D 1.5 + Kin 0.2	LD	60	FG	+++
	IN	55	FG	+++

(++) considerable callusing, (+++) profuse callusing, FG= Friable green, FB= Friable brown, LD= leaf disc, IN= Internodes.

Leaf disc and nodal segment were cultured on MS medium supplemented with various concentrations and combinations of BAP and NAA to induce callus (Fig. 1. A-B). Among them, the highest of 90% and 80% of cultures induced callus in the medium containing 1.0 mg/l BAP + 0.1 mg/l NAA for leaf disc and internodes, respectively. Those calli were friable brown in colour and very fast in growth. BAP with IBA was less effective than BAP with NAA. The highest of 40% and 30% cultures showed callus formation in medium having 1.5 mg/l 2, 4-D for leaf disc and internodes, respectively. 2, 4-D with BAP was more effective than 2, 4-D alone. The highest of 75% and 65% of cultures induced callus formation in medium having 1.5 mg/l 2, 4-D + 0.2 mg/l BAP for leaf disc and internodes explants, respectively. Maximum callusing rate of 90% and 80% was recorded leaf disc and internodes, respectively. It is clear that leaf explants proved to be best in term of callus induction. Role of auxin alone or combination with cytokine on callus proliferation was well reported. Similar kind of results was observed in *Coccinia grandis* (Thiripursasundari and Rao 2012), *Cucumis sativus* (Bergovet et al. 1989), *Momordica dioica* (Nabi et al. 2002) and *Momordica charantia* (Malik et al. 2009). There are many other reports on callus induction of different explants from different medicinal plants species when cultured auxin in combination with cytokine (Kashem and Rahman 2018, Gopalakishnan et al. 2009, Sharmin et al. 2014).

After induction of callus from leaf and internodes of *C. cordifolia* next step of the experiment was induction organogenesis in this callus tissue. Cytokinin works as a signalling molecule that activates totipotent cells of callus for shoot organogenesis (Adelberg et al. 1997). For this experiment, the callus was sub cultured on MS medium supplemented with different concentrations and combinations of BAP singly or with NAA and IBA for inducing organogenesis (Table 2). Twenty different concentrations of BAP with NAA combination were used for this purpose. Among them, 2.0 mg/l BAP with 0.5 mg/l NAA was found to be the best combination where 80% and 70% of calli regenerated into shoots for leaf disc and internode explants, respectively. The highest number of shoots regenerated per culture was 6.5 ± 0.10 and 5.6 ± 0.55 for leaf disc and internodes explants respectively (Fig. 1 C-G). Most of the cucurbitaceae members produced shoots from the callus culture in combination of auxin and cytokinins. Similar responses were also observed in other related plants including *Momordica dioica* (Nabi et al. 2002), *M. charantia* (Sultan and Bari Miah 2003) along with other medicinal plants including, *Alocasia amazonica* (Raju et al. 2022), *Arbus precatorius* (Biswas et al. 2007), *Scoparia dulcis* (Hassan et al. 2008). The results of the present study are also in agreement with the observation of *Solanum sisymbrifolium, S. nigrum* (Ara et al. 1993).

Adventitious shoots (0.5 -1.0 cm) were excised and cultured into hormone free as well as medium containing various concentrations of IBA, IAA or NAA mediated full and half strength MS medium (Table 3). Half strength MS medium produced the highest percentage of roots in almost every PGR concentration while 0.1 mg/l IBA developed the maximum number of roots of 6.2 ± 0.37 and 6.8 ± 0.10 in full-strength and half-strength nutrients, respectively (Fig. 1 H). Highest response rate of 95% was also observed at the lowest IBA concentration. For the other two auxins, similar observation was also recorded, although the root number and root length were lower than IBA and 0.2 mg/l concentrated medium for every auxin showed lower response rate and lower morphological attributes with moderate callus at the root-initiated region. Rooting is essential for micro-shoots for the ultimate development as plantlets, where the root system upholds the water and balance the water loss through poorly functioning stomata of leaves (Ehsandar et al. 2013). Therefore, maximum root induction efficacy was found at 0.1 mg/l IBA amended half-strength MS medium as it almost 95% micro-shoot regenerates roots (Table 3). The use of low salt MS medium for rooting in *in vitro* induced shoots is a very common practicefor *C. cordifolia* (L.) Cogn. as Roy et al. (2012) evaluated strong root induction of 82.4% at 0.5 mg/l IBA containing half-strength MS medium. Sekhawat et al. (2014) also reported the potentiality of half-salt strength in root formation and observed the best result in 2.0 mg/l IBA. Sundari et al. (2011) also reported IBA as an effective auxin for root formation, similarly IBA supremacy for root induction has also been reported in many others species of Cucurbitaceae (Hoque et al. 2007, Khalekuzzaman et al. 2012, Arciniega-Carreon et al. 2017).

Table 2. Regeneration of shoots from *in vitro* grown leaf and internodes derived callus of *Coccinia cordifolia* on MS medium.

Growth regulators (mg/l)	Types of Explants	Frequency of regeneration (%)	No. of shoots per culture ($\overline{X}$ ± S.E.)
BAP			
1.0	LF	30	3.5 ± 0.20
	IN	20	2.0 ± 0.15
1.5	LF	40	4.3 ± 0.35
	IN	35	3.8 ± 0.10
2.0	LF	60	5.0 ± 0.21
	IN	50	4.5 ± 0.30
2.0	LF	50	4.5 ± 0.20
	IN	45	4.0 ± 0.25
BAP + NAA			
2.0 + 0.2	LF	65	4.4 ± 0.31
	IN	55	4.2 ± 0.35
2.0 + 0.5	LF	80	6.5 ± 0.10
	IN	70	5.6 ± 0.55
2.0 + 1.0	LF	65	5.8 ± 0.25
	IN	60	5.0 ± 0.30
BAP + IBA			
2.0 + 0.2	LF	60	4.3 ± 0.30
	IN	50	3.9 ± 0.25
2.0 + 0.5	LF	70	5.1 ± 0.20
	IN	60	4.5 ± 0.50
2.0 + 1.0	LF	55	4.5 ± 0.10
	IN	45	4.0 ± 0.60

Well rooted plantlets were transferred into a thumb pot containing sand : garden soil : organic fertilizer (1:2:1) for hardening. The hardening process was applied for 2 weeks in growth room with a plastic cover on the pots to create a greenhouse-like condition (Sharmin et al 2013). The plants acclimatized to the *ex vitro* environment with 90% survival rate after a month (Fig. 1 I-J). For plant acclimatization to *ex vitro* condition root length and number is very important to uptake the nutrients and water in *C. cordifolia* plantlets (Sanavy and Moeini 2003). The planted plants acclimatized to *ex vitro* environment gradually after 15 days greenhouse condition and 85% of the plants survived in the condition after a month-long observation.

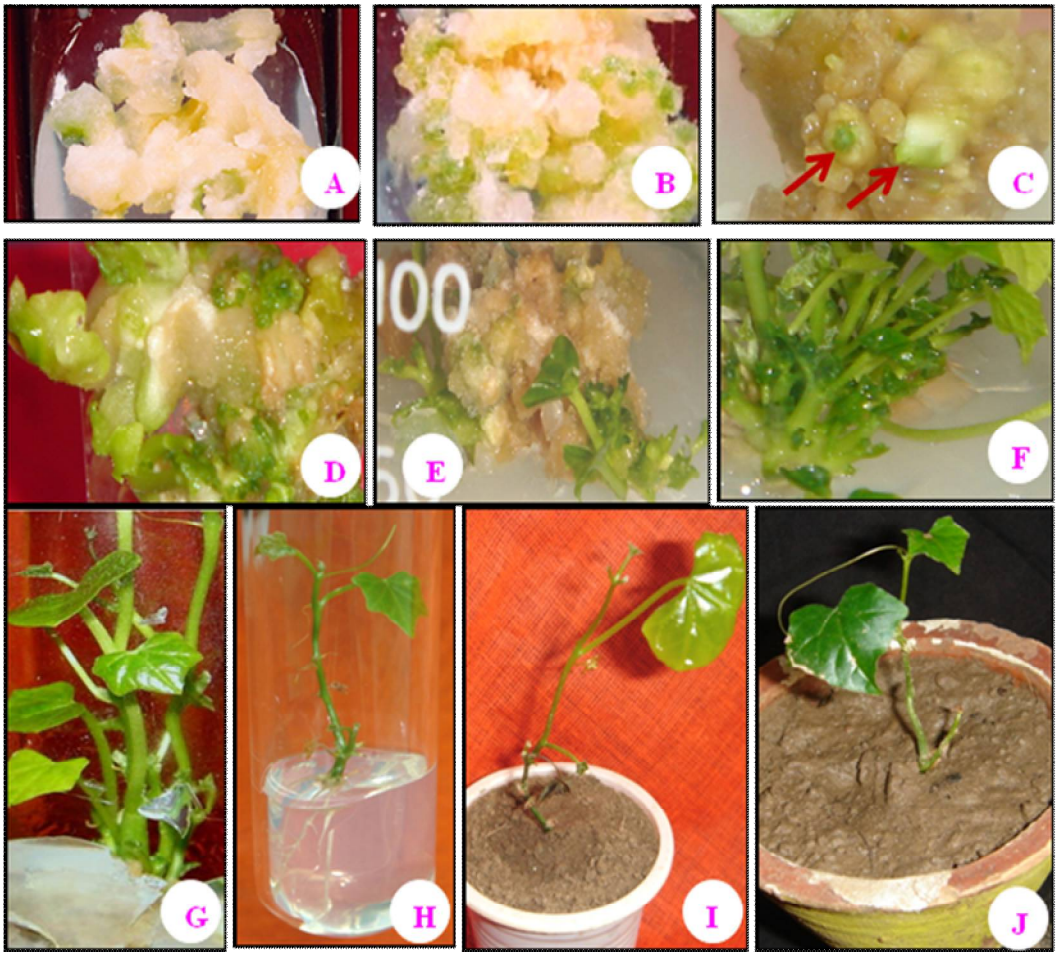

Fig. 1. Indirect organogenesis of plant regeneration from callus in *Coccinia cordifolia* (L.) Cogn. A: Callus induction; B: Proliferation of callus; C-E: Shoot regeneration in full-strength MS medium supplemented with 1.5 mg/l BAP and 0.1 mg/l NAA; F-G: Proliferated shoots; H: *In vitro* root formation in half strength MS medium supplemented with 0.1 mg/l IBA; I-J: Acclimatized plantlet.

During this study, a protocol was established for in *vitro* callus formation from leaf and internode explants of *C. cordifolia*. Upon manipulation of growth regulators, plantlets were regenerated from calli. The plantlets were rooted and acclimatized to the soil. The protocol would serve as the basis for biotechnological applications such as the development of transgenic plants and creation of somaclonal variation in this important medicinal plant.

Table 3. Effect of different concentrations of auxins on adventitious root formation from the *in vitro* grown micro-shoots cultured on full strength (MS) and half strength (MSS_1) of MS medium with 3% (w/v) sugar.

Types of auxin	Medium strength	Response of micro shoots rooted (%)	Number of root per microshoots ($\bar{X}$ ± S.E.)	Average length of root (cm) ($\bar{X}$ ± S.E.)	Callus formation at the cutting base
Control	MS	60	4.2 ± 0.70	2.3 ± 0.40	-
	MSS_1	70	5.2 ± 0.50	2.5 ± 0.10	-
IBA					
0.1	MS	90	6.2 ± 0.37	2.3 ± 0.10	-
	MSS_1	95	6.8 ± 0.10	2.8 ± 0.20	-
0.2	MS	75	4.5 ± 0.20	1.8 ± 0.20	+
	MSS_1	80	4.9 ± 0.20	2.1 ± 0.10	+
NAA					
0.1	MS	85	5.1 ± 0.30	2.0 ± 0.10	-
	MSS_1	90	5.5 ± 0.40	2.4 ± 0.25	-
0.2	MS	70	3.8 ± 0.10	1.6 ± 0.51	+
	MSS_1	70	4.1 ± 0.10	1.8 ± 0.51	+
IAA					
0.1	MS	65	4.0 ± 0.30	1.8 ± 0.40	-
	MSS_1	70	5.1 ± 0.30	2.1 ± 0.50	-
0.2	MS	55	2.3 ± 0.10	1.3 ± 0.10	-
	MSS_1	60	3.7 ± 0.10	1.6 ± 0.10	-

Notes: (-) represents no response; (+) indicates minor callusing.

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