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High efficient protocol for Callus induction and Regeneration of a medicinal plant *Orthosiphon stamineus*

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Abstract

Callus induction and indirect organogenesis protocol were developed using different explants of *Orthosiphon stamineus* such as leaf, petiole, and internode on MS medium supplemented with different plant growth regulators. For callus induction, MS medium with 2, 4-D, NAA and IBA alone or in combinations with BAP were used. High-efficiency callus induction was obtained (100%) in petiole compared with other explants at the concentrations of 5mg/l 2, 4-D hormone, and 99% callus induction was obtained in leaf and internode at the concentration of 4.0 mg/l 2,4-D hormone. Similarly, 100% callus induction was obtained at the concentrations of 4.0 mg/l NAA with 0.5 mg/l BAP combinations in petiole and internode whereas 99% of callus induction was obtained from the same concentration. The induced callus of leaf, petiole, and internode was transferred to shooting medium fortified with plant growth regulators at 5.0 mg/l BAP and 0.5 mg/l NAA. Among these, internode has shown maximum percentage of the shoot (60%) regeneration than petiole and leaf callus. Shoot regenerated plantlets were then transferred to ¼MS medium fortified with 1.0 mg/l NAA hormone results in high efficient rooting (20) in *vitro* plantlets without inducing basal callus. Further, root-induced healthy plantlets were subjected to hardening in the greenhouse and established in natural environmental condition with a survival rate of 95% without any morphological changes of true type plants. Hence the developed protocol was optimized to yield a high frequency of callus induction and regeneration in different explants of *O.stamineus* with different concentrations of plant growth regulator.

Keywords: *Orthosiphon stamineus*, leaf, internode, petiole, Callus induction, plant regeneration.

Introduction

Orthosiphon stamineus is a medicinal plant which belongs to Lamiaceae family. This plant is commonly called as kidney tea, java tea, and cat's whiskers. Extensively this plant leaf and meristematic shoots contain potassium ions that help to remove uric acid stones and sodium excretion from kidney and urinary bladder which was reported by Englert and Harnischfeger (1992) and Yuliana et al., (2009). Traditionally this herb is used to treat various kind of diseases such as gallstones, gout, edema, eruptive fever, hepatitis, and jaundice abdominal pain, kidney and bladder inflammation and highly used for diuretic activity (Awale et al., 2003; Eisai, 1995). Biologically active compounds and antioxidants are mainly present in leaves (Hollman and Katan, 1999: Matkowski, 2008; Chew *et al*., 2009; Pietta et al., 1998). This plant has already been reported to have its diverse novel secondary metabolites such as diterpenes, flavones and

triterpenoids (Koay and Amir, 2012). These plants well grew on wet soil and widely distributed both temperature and tropical region of south east Asia (Hsuan, 1986). This pharmacologically important medicinal plant has been exploited due to the commercialization of plant sources and flowers and flower buds were removed from plants for enhancing the accumulation of active constituents in the leaves. This continuous practice may result in the delayed seed setting and consequent consumption of plant become destroying the cultivation of this species. *O. stamineus* is erratically scattered and very rare in the field so that the protection of this plant is an important need to ensure its sustainable utilization (Rajendran et al., 2001). Plant tissue culture plays a major role in the preservation of rare medicinal plants and also enhances the production of secondary metabolites as like in *Solanum trilobite* (Priya, et al., 2014); *Citrullus colocynth* (Tanveer et al., 2012). Callus and suspension culture was used for the production of secondary metabolites from medicinal plants using cell culture technology (Mulabagal and Tsay et al., 2004). The main objective of the present study is to optimize a regeneration protocol for *O. stamineus*. This regeneration protocol can be used either as a micropropagation or to develop a platform for secondary metabolites productions through callus induction.

Materials and Methods

O. stamineus plants were collected from Palode region of Westernghat, Kerala and it was established and maintained in the herbal garden of the institute. Juvenile leaves, petioles and internodes were collected and washed under running tap water for 15 min and then the explants were washed with teepol solution for 8 min and transferred all explants in aseptic condition for surface sterilization with 0.1% (w/v) mercuric chloride solution for 3.0 min then the explants are rinsed three times with sterile double distilled water and subsequently sterilized explants were inoculated on Murashige and Skoog medium (Murashige and Skoog, 1962) containing 2.0% (w/v) sucrose, 0.6% (w/v) agar (pH 5.7). The medium fortified with different concentrations (0.1, 0.5, 1.0, 2.0, 4.0, 6.0 mg/l) of 2, 4-D and NAA, either single or in combination with BAP for callus induction. All *in vitro* cultures were incubated under dark condition at 25±1ºC for a week.

After proliferation of explants in the callus medium, it was transferred to culture room with 16-h photoperiod by cool-white fluorescent lamps with 48µmol.$m^{-2}s^{-1}$ photon flux density and 8-h dark condition at 25±1ºC.

All the explants were subcultured on regular intervals of every 20 days on MS medium and then green friable regenerative callus were transferred to callus regeneration medium fortified with both auxin and cytokinin combinations of different concentrations were analyzed and screened the suitable hormonal concentrations on MS medium. After 25 days of inoculation, data were recorded on the basis of a number of shoots regenerated per callus, shoot elongation and shoot length. The healthy equal length of above 3-5cm plantlets were transferred to different (1/4, 1/2 and full) strength of MS medium supplemented with NAA & IBA plant growth regulators for root induction and a total number of roots per plantlet was also recorded. Each experiment was repeated thrice with 10 replicates per treatment. Statistical analysis was performed utilizing SPSS 13 (SPSS Inc., Chicago, IL, USA) and Excel 2007 (Microsoft, Redmond, WA, USA) software. Data were analyzed using ANOVA to consider for significant and Least Significant Differences test was utilized to compare means at $p<0.05$ level.

Results

Effect of 2, 4-D on callus induction

Different range and degrees of callus induction was observed in all the three kinds of explants after 25 days of culture on MS medium fortified with various concentrations of 2, 4–D (1.0 – 6.0 mg/l) and the results were presented in Table 1. Among that concentration maximum cell proliferation (100%) was obtained at 4.0 & 5.0 mg/l of 2,4-D concentration within (11days) short duration of the time period with the soft friable texture of green white color, callus induction was observed for all the three different kind explants. At high concentration of 2, 4-D hormone, the callus induction was declined and turns hard texture with brown in color appearance was observed which leads to necrosis later.

Table 1: Effect of different concentration of 2, 4-D on MS medium for callus induction from leaf, internode and petiole explants of *O. stamineus*

Conc. of 2,4-D mg/l	Type of explants	Percentage of Callus induction ± S.D*	Time taken for callus response in days	Callus colour	Texture of callus
0.5	Leaf Internode Petiole	10.3±0.47 41.2±1.98 10.7±0.70	16 10 12	Green Green Green	Compact Compact Compact
1.0	Leaf Internode Petiole	19.7±0.45 64.4±0.80 30.9±0.94	14 10 12	Green Dirty white Pale green	Compact Friable Soft
2.0	Leaf Internode Petiole	64.3±1.40 73.8±2.52 50.6±1.44	12 13 10	Pale green Green Cream white	Friable Friable Friable
3.0	Leaf Internode Petiole	84.9±0.53 98.0±0.77 84.1±1.44	11 13 10	Green white Cream white Dirty white	Friable Friable Friable
4.0	Leaf Internode Petiole	99.6±0.80 99.1±1.30 99.9±0.30	10 07 09	Pale green Cream white Pale green	Friable Friable Friable
5.0	Leaf Internode Petiole	96.2±1.04 98.8±2.60 100±0.0	10 07 11	Dirty white Light brown Pale green	Soft friable Compact Friable
6.0	Leaf Internode Petiole	73.2±2.60 77.7±1.70 94.6±0.48	11 06 08	Brown Brown Cream white	Compact Compact Friable

*Values correspond to means ± SD (Standard deviation) of three replicates (5 explants / replicate).

Effect of NAA and BAP on callus induction

MS medium supplemented with different concentrations (0.5-5.0 mg/l) of NAA along with 0.5mg/l BAP used, the optimized the hormone concentration for the callus induction was 4.0 mg/l NAA+0.5 mg/l BAP which yielded the maximum percentage (100%) of callus induction in all three types of explants (Table 2). While the concentration of

NAA hormone increases, the efficiency of callus induction was gradually decreased and the explants became dry. The optimized concentration of NAA with BAP produced soft and friable pale green color callus which was suitable for the shoot regeneration.

Table 2: Effect of different concentration of auxin and cytokinin on MS medium for callus induction from leaf, internode and petiole explants of *O. stamineus*

Conc. of NAA+BAP (mg/l)	Type of explants	Percentage of Callus induction ± S.D*	Time taken for callus response in days	Callus colour	Texture of callus
0.5+0.5	Leaf	14.8±0.4	15	Green	Compact
	Internode	24.9±0.5	12	Green	Compact
	Petiole	10.1±0.3	10	Green	Soft &compact
1.0+0.5	Leaf	30.4±0.8	15	Pale green	Compact
	Internode	74.7±1.6	12	Pale green	Friable
	Petiole	24.3±1.5	10	Green	Friable
2.0+0.5	Leaf	81.0±1.3	11	Cream white	Friable
	Internode	95.0±0.6	10	White &green	Soft friable
	Petiole	64.8±0.6	08	Green	Soft friable
3.0+0.5	Leaf	94.7±1.6	10	Cream white	Friable
	Internode	99±1.0	10	Pale green	Friable
	Petiole	85.4±0.4	08	Pale green	Soft friable
4.0+0.5	Leaf	99.6±0.8	07	Green white	Friable
	Internode	100	07	Green white	Friable
	Petiole	100	07	Green white	Soft friable
5.0+0.5	Leaf	90.4±1.2	07	Dirty white	Compact
	Internode	96±0.4	07	Pale green	Friable
	Petiole	100	07	Pale green	Friable

*Values correspond to means ± SD (Standard deviation) of three replicates (5 explants / replicate).

Indirect shoot regeneration

The induced soft friable callus explants were subjected to the regeneration media for the development of whole plantlets using different concentrations of BAP (1.0-6.0 mg/l) along with 0.5 mg/l of NAA combinations in MS media. Among the different concentrations of plant growth regulators BAP 5.0 mg/l with NAA 0.5 mg/l has shown a maximum number of shoots regeneration (16.2) and shoot length (3.7 cm) (Table 3) obtained from internodal callus explants followed by shoot number of the petiole (12.8) and leaf (3.9) explants.

Rooting of shootlets

Healthy shoot cultures obtained from shoot regeneration medium were then transferred to the rooting medium for root induction. Among the medium and hormonal concentrations tested, the highest rooting percentage (89), root number (20) and root length (5.0 cm) was obtained in ¼MS medium supplemented with 1.0 mg/l NAA followed by IAA (16.7) and IBA (13.6) of same concentrations (Table 4). Basal callus with small rootlet formation was observed beyond the optimized plant growth hormone concentrations. Healthy rooted plantlets were transferred to hardening and the results showed 95% survival rate of all plantlets in natural environmental condition.

Table 3: Effect of different concentrations of auxin & cytokinin for shoot regeneration of *O. stamineus*

MS+PGRs	Concentration (mg/l)	Type of explants	Regeneration percentage	Mean No.of Shootlets regenerated ± S.D*	Mean height of shootlet (cm) ± S.D*
BAP+NAA	1.0+0.5	Leaf Internode Petiole	- - -	- - -	- - -
BAP+NAA	2.0+0.5	Leaf Internode Petiole	- - -	- - -	- - -
BAP+NAA	3.0+0.5	Leaf Internode Petiole	- - -	- - -	- - -
BAP+NAA	4.0+0.5	Leaf Internode Petiole	25 35 30	2.2 ± 0.4^{e} 3.2 ± 0.4^{de} 2.9 ± 0.3^{e}	2.0±0.2 2.7±1.4 2.4±0.3
BAP+NAA	5.0+0.5	Leaf Internode Petiole	40 **60** 50	3.9 ± 0.3^{d} $\mathbf{16.2\pm0.9}^{a}$ 12.8 ± 0.4^{c}	2.5±0.3 **3.7±0.8** 3.5±0.4
BAP+NAA	6.0+0.5	Leaf Internode Petiole	35 45 45	3.2 ± 0.4^{d} 14.8 ± 0.6^{b} 14.4 ± 0.5^{b}	2.1±0.1 2.5±0.6 2.0±0.2

*Values correspond to means ± SD (Standard deviation) of three replicates (5 explants / replicate); Dissimilar letters indicated significant difference between means within the treatment at $p<0.05$ level based on LSD mean separation; '-' indicates no response.

Table 4: Effects of different concentrations of auxins for root induction of *O. stamineus*

PGRs	Conc. (mg/l)	Frequency of root induction (%)	No. of days taken for rooting response	Mean No. of rootlets ± S.D*	Length of roots (cm) ± S.D*
IAA	0.5 1.0 2.0 3.0	65 80 50 20	10 10 09 15	16.5 ± 2.2^{b} 16.7 ± 2.8^{b} 9.5 ± 0.4^{d} 2.2 ± 0.7^{e}	3.7±0.8 3.7±0.6 2.2±0.2 1.3±0.2
IBA	0.5 1.0 2.0 3.0	50 70 40 10	10 10 12 17	8.0 ± 2.6^{d} 13.6 ± 3.5^{c} 3.4 ± 1.6^{e} 2.1 ± 0.1^{e}	3.0±0.4 3.8±0.6 1.9±0.2 1.2±0.2
NAA	0.5 1.0 2.0 3.0	70 89 76 10	07 07 10 15	9.4 ± 2.4^{d} $\mathbf{20.0\pm0.2}^{a}$ 10.0 ± 1.2^{d} 3.0 ± 0.8^{e}	3.8±0.5 **5.0±0.8** 2.0±0.3 1.2±0.1

*Values correspond to means ± SD (Standard deviation) of three replicates (10 shootlets / replicate). Dissimilar letters indicated significant difference between means within the treatment at $p<0.05$ level based on LSD mean separation

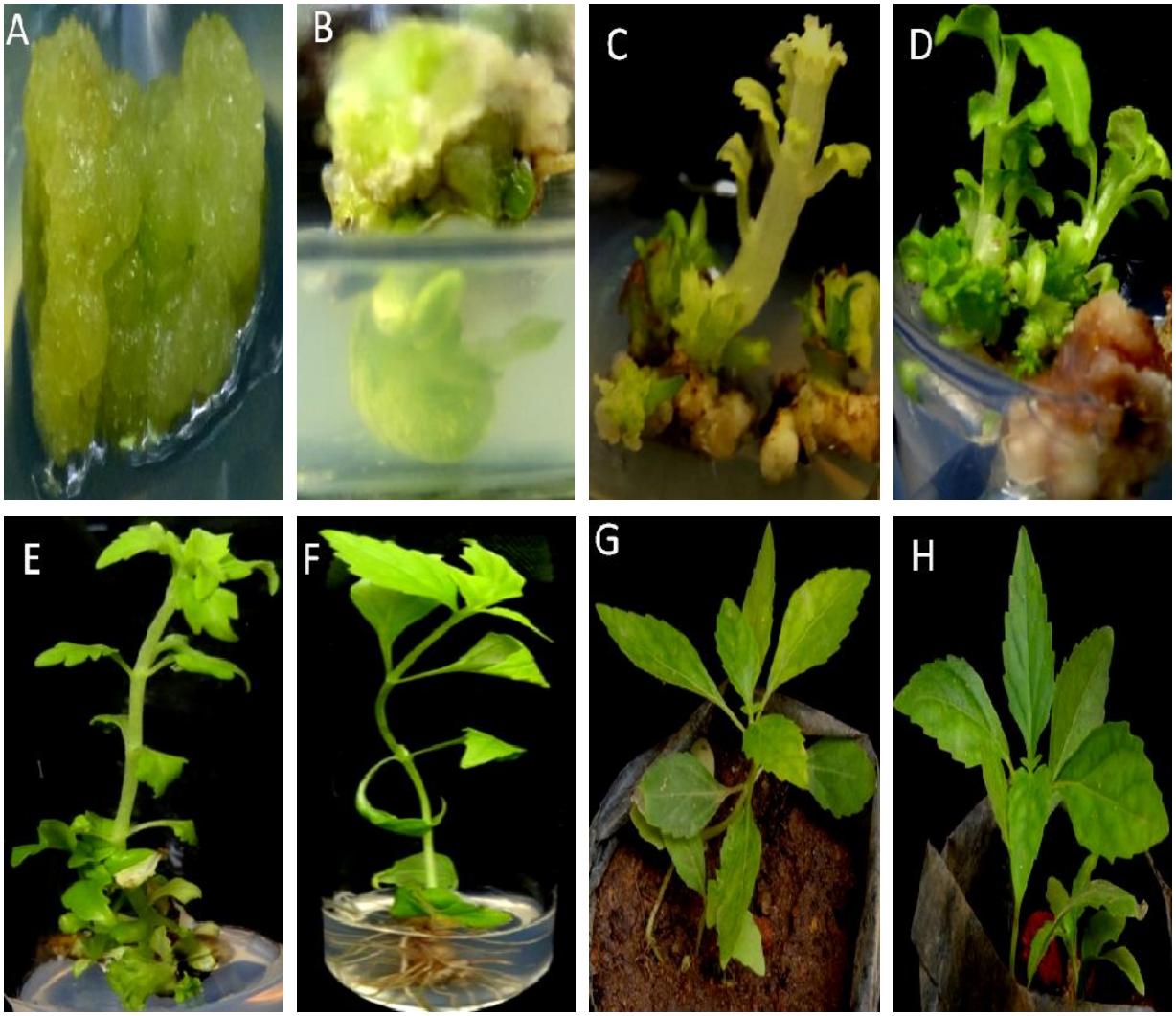

Fig. 1: Callus induction & regeneration of *Orthosiphon stamineus.* (A) *in vitro* Internode callus; (B) leaflets regeneration from callus; (C) *In vitro* shoot buds developed; (D) young regenerated shootlets become matured; (E) further elongated shootlets; (F) root induction of shootlet; (G & H) acclimatization of regenerated plantlets.

Discussion

Orthosiphon stamineus is an important medicinal plant traditionally used to treat kidney stone, gall bladder, and hyperglycemia diseases. The previous studies have been reported for the direct regeneration, cell suspension culture using different explants of this plant. However, there are only a few studies are reported about the callus regeneration from this plant. Hence, the present study was designed to evaluate the effect of various explants type, media, and plant growth regulator combinations to develop an *in vitro* callus induction and regeneration. Reshi et al., (2013) reported that callus production efficiency of NAA and BAP on *Orthosiphon aristatus.*

High yield of callus induction had been reported using 2, 4-D in some medicinal plants such as *Ipomoea obscura, Withania somnifera, Cardiospermum halicacabum* by Mungole et al., (2009). In the present study also high efficiency of callus induction was obtained from of all three type explants using 2, 4-D hormonal concentrations and subsequently used two different hormone combinations (NAA and BAP) for the screening of callus induction efficiency. BAP and NAA hormone combinations in MS medium was highly suitable for mass callus production from the leaf, internodal and petiole explants and identified different texture of soft, compact and friable proliferated cells with pale green to dark green color callus were formed. Callus induction frequency was higher in all medium containing 2,4-D than the medium with NAA and BAP this indicates 2,4-D hormone plays an important role in cell division (Sen et al., 2014). In *Ocimum sanctum* efficiency of

2, 4-D showed maximum callus formation (Lim et al., 2009). This study reveals that small root induction was found during callus formation on MS medium containing optimized concentration of NAA and BAP hormone only in the long term maintenance of cultures.

Callus induced by 2, 4-D was not suitable for regeneration of plantlets whereas NAA and BAP combinations based callus induction was suitable for shoot regeneration. Maximum regeneration percentage (20%) was reported by Guruchandran and Sasikumar, (2013) in *Stevia rebaudiana* at 0.5 mg/l BAP. However, in our studies, BAP with NAA combination showed 60% of callus regeneration using optimized plant growth regulators concentrations (BAP 5.0 mg/l + NAA 0.5 mg/l) for this plant. Regeneration of shoots from callus in MS medium supplemented with the only cytokinin was not suitable for *L. citriodora* (Mosavi, 2012). Here we obtained similar correlations of author reported BAP alone is not enough to obtained plant regeneration from callus and the combination of increasing BAP concentration with optimized NAA concentration induced shoot regeneration. *Lippia alba* showed an increase in BAP concentration leads to increase in shoot number (Gupta et al., 2000) Indirect shoot organogenesis through callus induction was obtained from leaf explants (Saritha et al., 2007) and (Agastian et al., 2006) in *Spilanthes acmella* and *Justicia gendarussa*. In contrast, leaf explants regeneration efficiency was less while compared to petiole and internode explants. At an optimized concentration of BAP with NAA improved regeneration efficiency but beyond the optimized concentration of cytokinin, auxin combination leads to the reduction in shoot number and some become necrosis.

Turker et al., (2009) reported similarly that optimized BAP concentration lead to increase in shoot regeneration percentage but more increasing concentration of BAP resulted in substantial reduction in regeneration efficiency. The combination of 2, 4-D and BAP in all media tested were failed to induce shoots regeneration from callus of *Orthosiphon stamineus* (Kamaludin Rashid et al., 2012), the present study reveals that MS medium supplemented with plant growth regulators such as BAP and NAA combination used for induction of indirect shoot regeneration from callus culture. Endogenous plant hormones already exist in plant tissues along with certain exogenous plant hormones combine together to induce shoot organogenesis; hence, the level of endogenous hormones in cultured explants and derived callus may be the most significant factor in shoot organogenesis (Lee and Huang 2013). In our study reveals that the level of regenerative callus induction into plantlets was done based on the combination of endogenous and exogenous plant growth hormones as per the authors (Lee et al., 2013) report. Auxins play an important factor in inducing somatic embryogenesis (Feher et al., 2003; Gaj, 2004). In the current study, explants such as petiole, internode followed by leaf showed an earlier response of callus induction in auxin-cytokinin combination along with small rootlets than with 2, 4-D alone. Nakano et al., (2000) and Krens et al., (2009) reported the efficiency of low regeneration might be caused by the long-term maintenance in culture. A plant growth regulator such as BAP alone or in combination with auxin has been reported earlier for shoot regeneration and multiplication in various *Brassica* species by Metz et al., (1995); Jin et al., (2000); Munshi et al., (2007); Maheshwari et al., (2011); Guo et al., (2005). Consequently, in this experiment, MS medium fortified with a different concentration ratio of BAP and NAA combinations was used for the screening of maximum number (16.2) of shoot regeneration/ internode derived callus while cultured on MS medium with optimized PGRs (BAP 5.0 mg/l+ NAA 0.5 mg/l) concentration.

The positive outcome of cytokinin (BAP or Kin or TDZ or 2iP or Zn) in combination with auxin (IAA, IBA & NAA) on shoot regeneration during organogenic segregation was found [Bhagya et al., (2013); Erisen et al., (2010); Mandal and Laxminarayana (2012); Perez-Jimenez et al., (2012); Wadl et al., (2011); Ghimire et al., (2010); Shen et al., (2007)]. In the present study BAP and NAA combination was tested and obtained the maximum shoot regeneration efficiency, which was noticed similar response to the above author's reports. The young mature shoots were transferred to the different strength of MS medium (MS, ½ MS, ¼ MS) fortified with different concentrations of NAA (0.5, 1.0, 2.0, 3.0 mg/l) moreover finally screened the optimized rooting media for development of whole plantlets to fulfill the efficient regeneration protocol for root induction. Among the different strength of MS medium and plant hormone concentrations, ¼ MS with 1.0 mg/l NAA showed maximum root induction. ½ strength MS medium was used for best root induction in cabbage and broccoli (Munshi et al., 2007); (Ravanfar et al., 2009). Different strength of MS medium without NAA resulted in fewer roots were risen above the surface of the shoots. These results are in parallel with the comments of

Ravanfar et al., (2009), while increased beyond the optimized concentration of NAA (1.0-2.0 mg/l) in the ¼ MS medium may induce callus formation at the base of the shoots with stunted roots (Munshi et al., 2007). Presently there is no perfect designed standard medium for growing seedlings of root studies, but many labs use complete or half-strength MS medium. Nitrogen source content in full strength MS medium consists of 21mM NH_4^+ and 40mM NO_3^-. From the authors reports the evidence of full-strength MS medium contain a high amount of nitrogen source was found to inhibit root growth (Dubrovsky et al., 2009). This study reveals that diluted strength of ¼MS medium with 1.0 mg/l NAA showed a maximum number of root induction. Less amount of nitrogen source containing (5.25mM $NH_4^+$and 10 mM NO_3^-) diluted strength of ¼ MS medium was enough to obtain a maximum number of rootlets within a short duration of time period. This medium may applicable for some other plants to develop maximum root induction of same or different family plants.

Conclusion

Efficient callus induction and plant regeneration was achieved on MS medium based on the type of explants (leaf, internode, and petiole) and the role of plant growth regulators in *in vitro* condition. Maximum callus induction (100%) was standardized using auxin concentrations of 2, 4-D 5mg/l and auxin-cytokinin combinations of NAA 4.0 mg/l and BAP 0.5 mg/l for all explants cultured on MS medium. Internode, petiole, and leaf derived green friable callus was subjected to shoot regeneration in MS medium fortified with a combination of BAP (5.0 mg/l) and NAA (0.5 mg/l) plant hormones showed to be an efficient combination for shoot induction. Shootlets were subjected to ¼ MS medium in addition to NAA (1.0 mg/l) plant hormone to increase the quantity and quality of roots as compared to other strength of MS medium with or without auxin concentration. Rooted plantlets were successfully acclimatized in a potting medium containing soil and perlite (3:1) and grew in greenhouse chamber. The survival rate of the regenerated plants was recorded 95% and no obvious phenotypic variations were observed among the regenerated plants. This reproducible protocol may be exploited for callus induction and regeneration of *O. stamineus* efficiently.

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