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RESEARCH ARTICLE

IN VITRO REGENERATION STUDIES OF OXALIS CORNICULATA FROM NODAL EXPLANTS

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ABSTRACT

An *in vitro* propagation protocol has been established for *Oxalis corniculata.* It belongs to the family Oxalidaceae is a extensively known plant for its traditional uses such as anthelmintic, anti-inflammatory and diuretic etc. The present study investigates the development of a simple, reproducible and efficient *in vitro* protocol for plant regeneration using nodal explants. *In vitro* shoot multiplication nodal explants of *O. corniculata* achieved on solid MS basal medium supplemented with different concentrations and combination of growth regulators such as Kn, BAP, NAA, IBA and IAA. Highest shoot regeneration and shoot number results was obtained on MS medium augumented with BAP (1.0mg/l) and in combination with NAA (0.5mg/l) with 96% of regeneration frequency. However the shoot length was less when compared to Kn. The maximum shoot length was obtained in Kn (2.0mg/l) with NAA (0.5mg/l). All the microshoots produced normal roots within four weeks of culture on the basal medium supplemented with auxins IBA and NAA. NAA was found to be best for rooting. Complete plantlets were then hardened, acclimatized and transplanted to natural conditions, where they exhibited 75% survivability.

Abbreviations

BAP	6-Benzylaminopurine
NAA	1-Naphthaleneacetic acid
IAA	Indole-3-acetic acid
IBA	Indole-3- butyric acid
Kn	Kinetin
MS	Murashige and Skoog

INTRODUCTION

Plants have been an essential source of medicine in ancient history apart from being a source of food, fibre and wood etc. Most of the drugs are active ingredients of plant metabolites. So it is necessary to conserve the endangered species through *in vitro* regeneration techniques. Plant tissue culture is a present application for high-speed propagation of plants. *In vitro* culture techniques is an essential module of the molecular breeding of plants and can direct to the generation of new plant cultivars in relatively petite period compared to the conventional breeding method. It can be used to conserve rare and in danger of extinction medicinal plants and proliferate them in short interval. *In vitro* shoot propagation involves commonly induction and development of shoot from explants like nodal, leaf, internodes and root, and this was followed the formation and development of roots in root induction medium. The tissue culture uses apparently for the production of compounds precious to the industry has long been a theme of interest for plant biotechnologists. Micropropagation has been viewed as an imperative technology for enhancing the competence of certain elite high acquiescent varieties, so as to enhance, production and productivity.

Oxalis corniculata. L belongs to family oxalidaceae, known as creeper wood sorrel or procumbent yellow sorrel is an herbaceous plant. Oxalis has rich medicinal uses where the whole plant was affluent in vitamin-C and used to treat scurvy. Secondary metabolites like tannins, flavones, glycoflavones, flavanols, palmitic acid, calcium and phenolic acids are copious in this plant. The stem is concised, pinkish brown, the leaves of wood sorrel are relatively edible and gives a tangy flavour of lemons. The trifoliate leaves are alternate with slender, heart shaped, leaflet blades having a discrete apical indention.

The flowers are yellowish in colour with 7-11 mm broad and having five petals. The fruit appears to be capsule, 1-1.5 cm long, cylindrical, pointed apically, and five ridged in cross section. The outline of the seeds are elliptical in nature, rounded apically, basally pointed, it looks like flattened in cross section, light brown and have a plane definitely diagonally grooved. *Oxalis corniculata* acquires vital activities like anti-oxidant, anti-cancer, anthelmintic, anti-inflammatory, analgesic, steroidogenic, and anti-microbial, anti-amoebic, anti-

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fungal, astringent, depurative, di-uretic, febrifuge, cardiorelaxan, stomachic and styptic and many other properties. it has been used to treat headache by the tribal people in koraput district of Orissa. And also as a folk medicine to cure skin diseases and in raw form is fine for digestion (Kohli 1993 and Das *et al.* 1987). It is also has healing properties to cure dysentery, diarrhoea, piles and skin diseases (Arya 1995 and Kirthikar *et al*. 1935). *Oxalis corniculata* has also showed perceptible antibacterial activity beside *E. coli*. (Unni *et al*. 2009).

MATERIALS AND METHODS

Collection of plant material

The plant material for the study of micropropagation was collected from the herbal garden, Dept of biotechnology, Dravidian University, Kuppam, Andhra Pradesh. The plant material preferred to be healthy, young, fresh and free of pest. The twigs were collected from the field and washed thoroughly under running water.

Surface sterilization of plant material

In order to inoculate the explants first it should be sterilised using the following chemicals. The explants collected from the field grown plants were washed thoroughly in running tap water for 10 minutes to get rid of soil particles. It was then washed with liquid detergent solution 5% (v/v) tween-20 for 20 minutes and then washed under running tap water (3 washes). In turn to get rid of fungal contamination, the explants were surface sterilized with 0.4% (w/v) bavistin a systemic fungicide (BASF India Ltd.,) the surface sterilized material was taken into sterilized room and sited in an inoculation chamber in a beaker containing double distilled water. Then the explants were treated with 70% (v/v) ethanol for 6o seconds. The final treatment of explants were surface sterilized with 0.1% (w/v) $HgCl_2$ (Merck india) for 1-3 minutes and at last they were rinsed with sterile double distilled water (3washes) before inoculation to remove traces of $HgCl_2$.

Culture medium

MS Medium (Murashige and Skoog, 1962) preferred for the inoculation explants material. it is the universally used media for *in vitro* propagation of plants. In the present study the explants were cultured on MS medium supplemented with various concentrations of growth regulators either alone or in combination where the p^H of the medium was attuned to 5.8 and gelled with addition of 0.8% agar (w/v). The medium molten was dispensed approximately 15 ml into culture tubes (25×150 mm) and closed with non-absorbent cotton plugs firmly. Autoclave for the medium was done at at 15lbs/sq inch pressure and 121^0C for 20 minutes.

Culture environment

After inoculation of explants in inoculation chamber, all the cultures were incubated in an *in vitro* culture room maintained at 26 ± 2^0C temperature with a relative humidity of 50-60% and photo period of 16 hours day light and 8 hours dark having light intensity provided 3000 lux by cool white fluorescent tubes. After 21 days of culture duration the cultures were transferred aseptically to fresh culture medium for the development of roots *in vitro.*

Data analysis

Visual consideration were recorded on the frequency in terms of number of cultures responds to nodal shoot proliferation, shoot development, number of shoots per explants, average length of the regenerated shoots, number of roots per shoot and average root length.

Statistical analysis

All the experiments were conducted with a minimum of 20 replicates and all assays were repeated at least three times. The experimental data was analysed statistically by one-way ANOVA using the DMRT (Duncan's multiple range test) ($P < 0.05$) and were represented as the mean average ± standard error (SE).

RESULTS AND DISCUSSION

Effect of cytokinins alone on in vitro plant regeneration of nodal explants of O. corniculata

Effect of BAP and Kn alone

MS medium supplemented with BAP and Kn was used alone with different concentrations (0.5-3.0mg/l). A difference in shoot regeneration was observed due to variable explants and exogenous level of growth regulator in medium. Number of shoots per explants among different concentrations and combinations of growth regulators were noticeably different. MS medium without growth regulator initiate shoot differentiation. But it resulted in the development of fewer shoots due to the existence of endogenous hormones. Similar observations were reported by Cavallini and Lupi (1987) and Paal *et al*. (1981).The data reveals that significant effect of BAP on the nodal cultures of this plant. After 10 days of culture inoculation shoot initiation and proliferation was observed (Fig. 1A). Nodal explants cultured on BAP at 1.0 mg/l alone reported maximum regeneration frequency (93%); mean shoot number (11.8±0.15) and decreased shoot length (3.1±0.1). Similar micropropagation protocol has been observed in *Rorippa indica* (Ananthi *et al*. 2011) (Fig. 1C). While least regeneration frequency (80%) in Kn at 2.0mg/l was produced havng mean shoot number (10.4±0.1) and increased shoot length (8.53±0.15) when compared to BAP alone.

Effect of cytokinins with combinations of auxins such as NAA, IBA, and IAA on in vitro plant regeneration of nodal explants of O. corniculata

Effect of BAP with NAA, IBA and IAA

The different combinations of cytokinins (BAP and Kn) and auxins (NAA, IBA and IAA) also experienced. The medium supplemented with BAP (1.0mg/l) and NAA (0.5mg/l) obtained maximum regeneration frequency (96%) and highest mean shoot number (24.6±0.28) with shoot length (3.83±0.1).

Whereas the combination of BAP (1.0mg/l) and IBA (0.5mg/l) favoured the mean shoot number (15.8±0.28) with shoot length (3.7±0.25). This was followed by the combination of BAP (2.0mg/l) and IAA (0.5mg/l) having mean shoot number (13.2±0.25) with shoot length (2.4±0.1) where the shoots appeared to be stunted growth. But the maximum and highest shoot length obtained in Kn (2.0mg/l) in combination with NAA (0.5mg/l). The shoot number were decreased with further increase in BAP concentration (>2.0mg/l). In the present study, BAP evoked best response for multiple shoot induction and proliferation from nodal explants than Kn. The effect of BAP and NAA on direct shoot regeneration was reported in *Gysophila paniculata* (Zukar *et al*. 1997) and *Spilanthes acmella* (Saritha and naidu *et al*. 2008) the enhanced rate of multiple shoot induction in cultures supplemented with BAP and NAA may be largely ascribed due to increased rate of cell division by cytokinin (BAP) in the terminal and axillary meristematic zone of explants tissue. Cells in this zone divide with the faster pace and thus produce large number of shoots.

Table 1 Direct shoot regeneration from nodal explants of field grown *O.corniculata* microshoots.

Plant growth regulators (mg/l)					Regeneration frequency (%)	Mean no. of Shoots/explants	Mean shoot Length (cm)
BAP	Kn	NAA	IBA	IAA			
0.5	-	-	-	-	78	8.4 ± 0.19^{ef}	3.5 ± 0.30^{gh}
1.0	-	-	-	-	93	11.8 ± 0.15^{p}	3.1 ± 0.1^{ef}
2.0	-	-	-	-	80	14.2 ± 0.2^{n}	2.36 ± 0.1^{c}
3.0	-	-	-	-	81	10.3 ± 0.1^{i}	2.1 ± 0.1^{bc}
-	0.5	-	-	-	73	7.5 ± 0.15^{c}	6.43 ± 0.32^{lm}
-	1.0	-	-	-	75	8.1 ± 0.10^{de}	6.63 ± 0.3^{mn}
-	2.0	-	-	-	80	10.4 ± 0.1^{i}	8.53 ± 0.15^{q}
-	3.0	-	-	-	77	7.4 ± 0.20^{c}	5.6 ± 0.10^{k}
0.5	-	0.5	-	-	70	20.3 ± 0.15^{r}	3.83 ± 0.15^{h}
1.0	-	0.5	-	-	96	24.6 ± 0.28^{t}	4.2 ± 0.25^{i}
2.0	-	0.5	-	-	85	21.8 ± 0.28^{s}	3.06 ± 0.1^{ef}
3.0	-	0.5	-	-	90	18.0 ± 0.5^{q}	2.7 ± 0.25^{de}
-	0.5	0.5	-	-	79	10.3 ± 0.15^{i}	4.3 ± 0.15^{i}
-	1.0	0.5	-	-	85	11.2 ± 0.25^{k}	6.16 ± 0.28^{l}
-	2.0	0.5	-	-	90	15.3 ± 0.15^{o}	8.3 ± 0.15^{q}
-	3.0	0.5	-	-	75	12.4 ± 0.2^{l}	7.3 ± 0.10^{p}
0.5	-	-	0.5	-	86	12.4 ± 0.1^{l}	3.2 ± 0.10^{fg}
1.0	-	-	0.5	-	90	15.8 ± 0.28^{p}	3.7 ± 0.25^{h}
2.0	-	-	0.5	-	85	13.4 ± 0.20^{m}	2.7 ± 0.25^{de}
3.0	-	-	0.5	-	83	$10.6\pm.0.28^{ij}$	2.26 ± 0.20^{c}
-	0.5	-	0.5	-	80	8.3 ± 0.15^{e}	5.63 ± 0.15^{k}
-	1.0	-	0.5	-	85	9.7 ± 0.25^{h}	6.26 ± 0.25^{lm}
-	2.0	-	0.5	-	90	12.2 ± 0.25^{l}	7.26 ± 0.25^{p}
-	3.0	-	0.5	-	80	8.8 ± 0.26^{f}	4.8 ± 0.26^{j}
0.5	-	-	-	0.5	92	9.8 ± 0.26^{h}	1.43 ± 0.1^{a}
1.0	-	-	-	0.5	94	10.2 ± 0.30^{i}	1.83 ± 0.28^{b}
2.0	-	-	-	0.5	95	13.2 ± 0.25^{m}	2.4 ± 0.1^{cd}
3.0	-	-	-	0.5	85	11.0 ± 0.15^{jk}	1.8 ± 0.26^{ab}
-	0.5	-	-	0.5	79	7.8 ± 0.26^{cd}	4.3 ± 0.1^{i}
-	1.0	-	-	0.5	87	9.3 ± 0.26^{g}	6.83 ± 0.28^{o}
-	2.0	-	-	0.5	94	6.9 ± 0.05^{b}	5.7 ± 0.15^{k}
-	3.0	-	-	0.5	75	5.8 ± 0.28^{a}	5.0 ± 0.50^{j}

Data represent treatment means ± SE followed by different letter (s) within a column indicate significant differences according to ANOVA and DMRT test (P <0.05).

Effect of Kn with NAA, IBA and IAA

Direct shoot regeneration was tested on Kn alone and in combination with NAA, IAA and IBA. The most favourable concentration for direct shoot induction using Kn (2.0mg/l) was in combination with NAA (0.5mg/l) produced (15.3±0.15) mean number of shoots and the maximum shoot length (8.3±0.15). But the number of shoots was less in Kn when compared to BAP. In Kn and IBA supplemented medium the maximum shoot number (12.2±0.25) was obtained with mean shoot length (7.26±0.25) at Kn (2.0mg/l) and IBA (0.5mg/l). This in turn followed by Kn and IAA supplemented medium with maximum shoot number (9.3±0.26) was obtained with mean shoot length (6.83±0.28) at Kn (1mg/l) and IAA (0.5mg/l). The number of shoots was decreased in Kn with further increase in the concentration. Enhancement of shoot regeneration in the presence of auxins and cytokinins has been reported in several species such as *Echinaceae purpurea* (Koroch *et al*. 2002), *Catalpa ovate* (Lisowska and wysokinska 2000) and *Vinca rosea* (Haq *et al*. 2013).

The varied effects of BAP and Kn shoot growth may be due to differential mode of action of BAP and Kn in shoot development. Cytokinins such as BAP and Kn are well versed to promote cell division and cell expansion in plant development. Many studies reveals that kinetin is effectual in stimulating shoot elongation but ineffective for shoot multiplication. In contrary BAP is known to be very effective in revitalizing shoot multiplication rather than inducing shoot elongation (Bon *et al*. 1998; Sinha *et al*. 2000; Rajeswari and paaliwal 2006).

Figure 1 Direct shoot regeneration from nodal explants of field grown *O.corniculata*.

A. Intiation of shoot bud from nodal bud explants after 7 days of inoculation on MS medium + BAP (1.0mg/l).
B. Shoot intiation from nodal explant on MS medium + BAP (1.0mg/l) and NAA (0.5mg/l).
C. Shoot multiplication from nodal explant on MS medium+ BAP (1.0mg/l) and NAA (0.5mg/l).
D. Elongated multiple shoots regenerated from nodal explant on MS medium + BAP (1.0mg/l) and NAA (0.5mg/l).
E. Intiation of roots from the regenerated shoots *in vitro* on MS medium + NAA (2.0mg/l).
F. Plantlet showing elongated root system.
G. Hardened plant in polythene bag containing soil and vermicompost in 1:1 ratio.
H. Plantlet in field conditions.

Table 2 Root organogenesis of microshoots from field grown *O.corniculata* supplemented with various concentrations of IBA and NAA on full strength MS medium.

Plant growth regulators (mg/l)		Regeneration frequency (%)	Mean no. of Roots/explant	Mean Root Length (cm)
IBA	NAA			
0.5	-	80	10.4±0.15[a]	3.4±0.20[b]
1.0	-	85	15.6±0.32[c]	5.2±0.25[d]
2.0	-	95	18.4±0.17[f]	4.2±0.05[c]
3.0	-	90	16.3±0.15[d]	3.5±0.40[c]
-	0.5	82	11.9±0.36[b]	4.4±0.20[c]
-	1.0	87	16.5±0.25[d]	6.2±0.25[e]
-	2.0	98	20.5±0.20[g]	5.3±0.15[d]
-	3.0	93	17.3±0.15[e]	3.1±0.1[a]

Data represent treatment means ± SE followed by different letter (s) within a column indicate significant differences according to ANOVA and DMRT test (P< 0.05).

Rooting of in vitro regenerated shoots

The *in vitro* developed shoots were aseptically excised and fixed on MS medium supplemented with different auxins (NAA, IBA and IAA) for rooting. In our study it showed that NAA provoked the best response in rooting having maximum number of roots (20.5±0.20) at 2.0mg/l while compared to IBA (2.0mg/l) Supplemented medium where induced roots (18.4±0.17) respectively. And our study shows that the number of roots drastically decreased with further increase in concentration (> 2.0mg/l) table 2. The results obtained are consistent with *Daucus carota* (Pant and manandhar) and *Lycopersicum eculentum* (Bekhi lesley 1976 and Kartha *et al.* 1976).

Acclimatization and transfer of plantlets to the soil

Studies were performed on rooting of *in vitro* regenerated shoots after four weeks later. *In vitro* regenerated plantlets with sufficient roots were taken out from the culture tubes cautiously and washed to take away the surplus of agar. Roots which were obtained in *in vitro* regenerated plantlets were then acclimatized to pots containing soil and vermicompost in equal proportions.

Then it was maintained in polythene membrane for about 8-10 days. After that the extant plants placed in pots and endorsed to grow under green environment for hardening. Finally the plants were transferred to field conditions with maximum survivability.

CONCLUSION

Oxalis corniculata is a wonderful plant having scope of different activities. A proficient protocol has been developed for successful micropropagation and multiplication of an important medicinal plant *O.corniculata*. The *in vitro* regeneration protocol with efficient shoots, trouble free rooting of microshoots and the plantlets were without difficulty, acclimatized to the outer environment and undergoing usual physiological development. It is highly beneficial for the production of uniform source of *O. corniculata* in series to promote wider biotechnological applications.

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