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In vitro Flowering and Production of Seeds in *Oxalis corniculata* L. - an Important Medicinal Plant

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Key words: Plant growth regulators, *In vitro* flowering, *Oxalis corniculata,* production of seeds, medicinal plant.

Abstract

The multiple shoots (14.2) from *Oxalis corniculata* differentiated through bud breaking on MS medium with 2.0 mg/l BAP. The regenerated shoots were further sub-cultured on MS medium containing 1.0 mg/l BAP and 0.5 mg/l NAA. Vegetative to reproductive phase transition occurred in the cultures affected by plant growth regulators (PGRs), sucrose concentrations, strength of MS salts and light. Flower buds initiated from the regenerated shoots on half strength of MS salts with 1.0 mg/l Kn + 0.5 mg/l NAA and 3% sucrose. These flower buds opened under low light (10-15 µmol m^{-2} s^{-1}) condition with 18h/6h photoperiod within 3 weeks of culture initiation. The shoots did not flower in the dark. Though smaller in size, the *in vitro* generated flowers were morphologically comparable to the natural flowers. Fruits were set in the cultures within 5 weeks.

Introduction

Oxalis corniculata L. is a medicinally important plant also known as creeping wood sorrel or sleeping beauty. The flora appears to be very delicate, low-growing, herbaceous plant of Oxalidaceae Family. It is expansively found in the regions of tropical and sub-tropical regions of the world. These woody sorrels are grown all over the year (perennial) and with tap-rooted herbs. The herb is bushy or mat-forming and 0.1-0.5 m tall. The arrangement of foliage is alternate along the stems. The leaves are in the form of tri-foliate with thin, heart shaped. A single long stalk arises from the axils of the leaf, in which three flower stalks extends each one with a single flower. The flowers are 7-11 mm wide and have yellow colour with five petals. Fruit contains loculicidal capsule (1-1.5 cm), cylindric, pointed apically and five ridged in cross section and are oval in shape.

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This is an important medicinal plant has several uses such as anti-inflammatory, refrigerant and antiscorbutic (Kirtikar K. R. and Basu 1988), wound healing activity (Ashokk et al. 2010), Anti-implantation and abortifacient activity, (Taranalli et al. 2004), Anti-diabetic activity (Sharangouda and Patil 2007), anti-cancer activity (Jyothi et al. 2011), anti-nociceptive activity (Kathiriya et al. 2010), antioxidant activity (Borah et al. 2012). It contains phytochemical constituents such as flavanoids, tannins, phytosterols, and volatile oils. The foliage contains flavanoids, isovitexine and vitexine -2" -o- beta- D-glucopyrunoside. It has an affluent source of essential fatty acids like palmitic acid, oleic, linoleic and stearic acids.

In vitro culture techniques provide an ideal experimental system in order to understand the mechanisms involved in flowering. However, *in vitro* flower bud induction is produced in few species under special conditions. This induction relies on several features such as genetic, hormonal and trophic factors and seems to be the result of the repression of growth genes and the activation of those responsible for flowering process (Rkhis et al. 2006). Effect of BA on high-frequency *in vitro* flowering in *Dianthus chinensis* L. is also reported (Sreelekshmi and Siril 2019). Stephen and Jayabalan (1998**)** noticed that the phytohormone gibberellic acid has a significant role in the induction of flowering. *In vitro* and *in vivo* flowering response in chicory was reported by Demeulemeester and De proft (1999). Flower induction *in vitro* was succeeded by many researchers in juvenile *Dendrobium* hybrids (Sim et al. 2007; Tee et al. 2008)**.** Many Physico-chemical factors influence the *in vitro* flowering mechanism in flora. Carbohydrates, growth regulators, light and pH of the culture medium are the important factors for *in vitro* flowering. This *in vitro* flowering provides information to evaluate the physiology and interaction of phytohormones, phenolics and sugars.

The principle involved in the Rapid generation technology (RGT) is that of growing miniature plants in a synthetic nutrient medium under controlled conditions and allocated them to produce a few flowers where they developed into seeds that are harvested prior to normal seed maturity, hence reducing the time period of the breeding cycle (Mobini et al. 2015). It was reported by Ali et al. (2008) the seed setting was improved in *Vigna radiata,* after addition of the exogenous plant growth regulators. It was well documented by Mamidala and Nanna (2009) that the flower induction and fruiting had been observed in the combination of IAA (0.5µM/l) and Zeatin (9.1µM/l) in *Lycopersicon esculentum*. *In vitro* flowering and formation of pod has been achieved in *Lens culinaris* (Sarker et al. 2012). Reduction in the time duration of *in vitro* and seed setting is an advantage which in turn reduces the length of the period required for the completion of life cycle (Dimri et al. 1977). *In vitro* seed production is very low, and its genetic purity is of high value. However, reports on *in vitro* fruiting and seed set are very rare. Our present study is to develop an efficient method for *in vitro* propagation for multiple shoots of *O. corniculata* by using nodal explants. We would like to optimize various physical and chemical factors that can influence the *in vitro* flowering and production of seeds in *O. corniculata*.

Materials and Methods

A healthy plant (Fig. 1A) was taken from Dravidian Herbal garden, Department of Biotechnology, Dravidian University, Kuppam-517426, Andhra Pradesh, India during the period from July to September. Axillary shoots were collected from 1 to 2 month old field grown plant. The nodal segments of 6-8 mm length representing 2-3 nodes were used in the present study. All nodal sections were cleaned with Ch1% (v/v) Teepol and kept under running water for 15 min. The explants were first subjected to fungicide 0.1% (w/v) bavistin (BASF India Limited, Mumbai, India) for 5-7 min and then surface sterilized with 0.1% (w/v) solution of $HgCl_2$ (Hi-Media, India) for 3 min under aseptic conditions. Then these explants were washed 5-6 times with sterilized distilled water.

MS medium (Murashige and Skoog 1962) with 3% (w/v) sucrose and 0.8% (w/v) agar-agar (Bacteriological grade, Qualigens Fine Chemicals, Mumbai, India) was used as the basic medium. The pH of all media was adjusted to 5.8 with using 1 N NaOH or 1N HCl before autoclaving at 121°C and 15 psi for 15 min. All cultures were maintained at 28 ± 2°C and 60% RH under a 15 hrs photoperiod with light supplied by white fluorescent tubes at an intensity of 40-50 µmol m^{-2} s^{-1}.

Surface sterilized explants were inoculated on MS medium provided with various concentrations of cytokinins (0.5-3.0 mg/l of BAP or 0.5-3.0 mg/l of Kn) were incorporated in culture media for multiple shoot induction. *In vitro* regenerated shoots were cut into 2.5-3.0 cm long shoot segments (each with 1-2 nodes) and sub-cultured by making a clump of 4-5 shoots and transferring shoot clumps on MS medium with various concentrations of cytokinins (0.5-3.0 mg/l of BAP or 0.5-3.0 mg/l of Kn) alone or in combination with auxins such as (0.5 mg/l each of NAA, IBA and IAA). Multiplication of shoots by sub-culturing was performed at 20 days intervals.

Well-developed individual shoots (4-5 cm long) maintained on MS medium with 2.0 mg/l BAP were selected for *in vitro* flower induction. These shoots were transferred to various nutritional and environmental conditions to get optimum conditions for flowering. The percentage of *in vitro* flowering/fruiting was calculated as the number of flowering/fruiting shoots out of the total number of cultured shoots. The following factors were studied for *in vitro* flowering and fruiting.

To evaluate the effect of PGRs on *in vitro* flowering, shoots collected from the multiplication medium were transferred to MS medium supplemented with various concentrations (1.0-3.0 mg/l) of kn, BAP alone or combination of with NAA, IAA (0.5 mg/l). To evaluate the effect of sucrose concentration on *in vitro* flowering, the individual shoots were transferred to MS medium with sucrose concentration added (3%) keeping other parameters (PGRs, Agar) constant. Similarly, individual shoots were transferred to the medium having other parameters at constant level but differing in strengths of MS salts (full, half) to get optimized conditions for *in vitro* flowering as well as fruiting. All the cultures were maintained under two different light intensities, one in normal light

(40-50 µmol m^{-2} s^{-1} PFD) and the other in low light (10-15 µmol m^{-2} s^{-1} PFD)with 18 h light and 6 h as well as in dark.

The *in vitro* produced flowers self-pollinated and produced fruits. After the maturation of the *in vitro*-produced fruits, the seeds were collected and tested for viability by two means. (i) Seeds were inoculated on PGR free MS medium under aseptic condition and placed in dark at 26°C and (ii) Seeds were placed on a layer of What-man No.1 filter paper moistened with distilled water in a petri dish in dark room at 26°C. All the experiments in the present study were conducted and repeated thrice with a minimum of 30 replicates per treatment. One replicate means one culture vessel. The significance of differences among means was carried out using Duncan's multiple range tests (DMRT) at $P \leq 0.05$. The results are expressed as means ± SD of three experiments and subjected to one-way analysis of variance (ANOVA) using SPSS V.17 (SPSS, Chicago, USA).

Results and Discussion

Irrespective of the types and concentrations of cytokinins such as BAP and Kn singly and in combination with NAA, IBA and IAA in MS medium, shoots regenerated from the nodal explants within 10-15 days of inoculation. MS medium augmented with different concentrations and combinations of PGR are used. Multiple shoots were obtained from nodal bud explants on MS medium amended with various concentrations of BAP (0.5-3.0 mg/l) and Kn (0.5-3.0 mg/l). The results showed that the variation in regeneration frequency, mean shoot number and mean shoot length (Table 1). The maximum frequency and number of multiple shoots was more on MS medium amended with BAP over Kn. The maximum number of multiple shoots (14.2 ± 0.2) with regeneration frequency (80%) was obtained at the concentration of BAP 2.0 mg/l (Fig. 1B and C). Induction of shoots using

(a) Mother plant used as source of explants. (b) Multiple shoot induction from nodal segments on MS medium supplemented with 2.0 mg/l BAP + Additives. (c) Shoot multiplication on MS medium supplemented with 1.0 mg/l BAP mg/l. (d and e) Flowering shoots on MS medium with 8 mg/l sucrose + 1.0 mg/l BAP + NAA 0.5 mg/l. (f) *In vitro* rooting on half strength MS medium supplemented with 0.5 mg/l of IBA. (g) *In vitro* flowering on medium with half strength of MS salts and 08 mg/l sucrose supplemented with 1.0 mg/l Kn + 0.5 mg/l NAA maintained under low light (10-15 µmol m^{-2}/s PFD) conditions. (h) *In vitro* Multiple flowers induction on MS medium supplemented with 2.0 mg/l Kn + 0.5 mg/l NAA maintained under low light (10-15 µmol m^{-2}/s PFD) conditions. (i) *In vitro* fruiting on medium with half strength of MS salts and 3% sucrose supplemented with 2.0 mg/l Kn + 0.5 mg/l NAA maintained under low light (10-15 µmol m^{-2}/s PFD) conditions. (j) Flower produced under *in vitro* condition. (k) Flower produced in nature. (l and m) Fruit produced *in vitro* and *in vivo* respectively. (n) Germination of seeds produced under *in vitro* conditions on MS medium without PGRs.

Table 1. Effect of different cytokinins such as BAP and Kn in combination with auxins NAA, IBA and IAA added to MS medium for the induction of multiple shoots from nodal explants of *O. corniculata*.

Plant growth regulators (mg/l)					Regeneration frequency (%)	Mean no. of shoots/explants (Mean ± SE)	Mean shoot length (cm) (Mean ± SE)
BAP	Kn	NAA	IBA	IAA			
0.5	-	-	-	-	78	8.4 ± 0.19^{ef}	4.16 ± 0.20^{hi}
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 ± 0.1b^{c}
-	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^{p}
-	3.0	-	-	-	77	7.4 ± 0.20^{c}	5.6 ± 0.10^{k}
0.5	-	0.5	-	-	70	20.3 ± 0.15^{r}	3.36 ± 0.20^{f}
1.0	**-**	**0.5**	-	-	**98**	**24.6 ± 0.28^{t}**	4.8 ± 0.26^{j}
2.0	-	0.5	-	-	85	21.8 ± 0.28^{s}	3.0 ± 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^{p}
-	3.0	0.5	-	-	75	12.4 ± 0.2^{l}	7.3 ± 0.10^{d}
0.5	-	-	0.5	-	86	12.4 ± 0.1^{l}	3.2 ± 0.10^{f}
1.0	-	-	0.5	-	90	15.8 ± 0.28^{p}	3.7 ± 0.25^{g}
2.0	-	-	0.5	-	85	13.4 ± 0.20^{m}	2.7 ± 0.25^{de}
3.0	-	-	0.5	-	83	10.6 ± 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^{o}
-	3.0	-	0.5	-	80	8.8 ± 0.26^{f}	3.83 ± 0.15^{gh}
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^{b}
-	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^{n}
-	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}

Culture condition: 25 ± 2ºC. 2 weeks of culture. SE = Standard error of mean; Values are the mean of ten replications. The values with the same superscript in each column are not significantly different at $p \leq 0.05$.

BAP was reported in *Withania somnifera* (Manickam et al. 2000). The number of multiple shoots decreased with further increase (>3.0 mg/l) in cytokinin concentration. Similar findings were reported by Komalavalli and Rao (2000) in *Gymnema sylvestris.* It was found that more number of multiple shoots was induced in BAP when compared to Kn (Thoyajaksha and Rai 2001). Where the highest shoot length (8.5 cm) with multiple shoots (10.4 ± 0.10) in Kn alone at the concentration of 2.0 mg/l**.**

Fig. 1. *In vitro* flowering and fruiting of *Oxalis corniculata.*

Maximum regeneration frequencies were obtained in all the concentrations used. Mass propagation of shoots obtained from repeated sub-culturing of explants derived from aseptic shoot cultures is another method practiced in many plant systems (Krishnan and Seeni 1994). Proper ratio of auxins and cytokinins is crucial for morphogenesis, leads to the development of complete plantlets (George and Sherrington 1984). Multiple shoots can be improved by repeated sub-culturing at regular intervals by excising the shoots and transferring them to the fresh medium with (21-25 days) passage times. The regeneration frequency and the number of multiple shoots increased on MS medium supplemented with BAP in combination with NAA. The highest number of multiple shoots (24.6 ± 0.28) (Table 1 and Fig. 1D & E) was achieved after sub-culturing on MS medium containing BAP (1.0 mg/l) and NAA (0.5 mg/l). The shoots formed were

healthier with broad leaves. Similar findings observed in *plumbago rosea* (Harikrishnan and Hariharan 1996). In BAP and IBA amended medium, high frequency and highest number of multiple shoots (15.8 ± 0.28) obtained at the concentration of 1.0 mg/l BAP and 0.5 mg/l IBA. The number of shoots and frequency were less when compared to BAP and NAA. In BAP and IAA supplemented medium the maximum number of shoots (13.2 ± 0.25) obtained at the concentration of 2.0 mg/l BAP and 0.5 mg/l IAA. Kn was ineffective in producing multiple shoots in *O. corniculata* when compared to BAP. Superiority of Kn, induced multiple shoot elongation with increased internodal length when compared to BAP. But the shoot length was decreased with increased concentrations of BAP. Similar findings were also observed in *Withania somnifera* (Saritha and Naidu 2007).

In the present study, BAP evoked a best response for multiple shoot induction and proliferation. Direct shoot organogenesis was experienced in Kn with the combination of NAA, IBA and IAA. The maximum number of shoots (15.3 ± 0.15) and shoot length (8.3 cm) was obtained using Kn (2.0 mg/l) with the combination of NAA (0.5 mg/l). In Kn (2.0 mg/l) and IBA (0.5 mg/l) amended medium, number of shoots (12.2 ± 0.25) was obtained (Fig. 1F). This is followed by Kn (1.0 mg/l) and IAA (0.5 mg/l) supplemented medium, where the number of shoots (9.3 ± 0.26) were obtained. Many studies reveal that kinetin is effectual in stimulation and shoot elongation but ineffective for shoot multiplication. On the contrary, BAP is known to be very effective in revitalizing shoot multiplication rather than inducing shoot elongation (Rajeswari and Paliwal 2006**)**.

The multiple shoots which are produced from axillary buds showed *in vitro* flowering on MS medium augmented with various combinations and concentrations of cytokinins (BAP and Kn) and auxins (NAA and IAA) in an incubation period of 18 hrs light and 6 hrs dark. Exogenous hormones have been added up to the endogenous contents results in increasing the hormonal level needed for flowering. Kn inhibited *in vitro* flowering in *Chamomile*. (Kintzyos and Michaelakis 1999). Contrary to the above statement, in the present study, *in vitro* flowering and fruiting has been induced only in the presence of Kn and NAA/IAA combination. The presence of carbon sources in culture medium is necessary for floral stimulation (Singh et al. 2006). The floral buds developed after 4 weeks of sub-culture, and the flowers are small and yellowish colour, when compared to field grown flowers. Induced flower buds remain unopened and failed to attain full bloom. Maximum number of flowers/culture (**3**) is obtained in the combination of Kn (2.0 mg/l) and NAA (0.5 mg/l) **(**Table 2**)** (Fig. 1H). It was surprisingly observed that, *in vitro* fruiting was achieved and the matured seeds wilted within the tube. The seeds obtained from *in vitro* are germinated within the tube and when the sprouted *in vitro* seeds sub-cultured, they formed multiple shoots but the shoots are very tiny. Factors effecting *in vitro* flowering and fruiting of *Pisum sativum* was reported by Franklin et al. (2000**)**. Cytokine is a common requirement for *in vitro* flower formation (Scorza 1982**)**. Flower formation was not observed in the combination of BAP-NAA/IAA. In contrast, flowering response was reported in plants like *Nicotiana tabacum* (Smulders 1990) and *Zea Mays* (Mandal et al. 2000) in the presence of cytokinin such as BAP.

The presence of auxins such as IAA/NAA has certain role in promoting *in vitro* flowering and fruiting in combination with Kn. The inclusion of Kn appears to be necessary for the induction of *in vitro* flowering in *Scoparia dulcis* (Mahender et al. 2008). It was reported by Sheja and Mandal (2003) that *in vitro* flowering and fruit formation was observed in *Lycopersicon esculentum* at elevated levels of endogenous auxins. *In vitro* flowering was considered as a complex process, which was controlled by the internal and external factors where its activation under *in vitro* conditions is very rare (Stephen and Jayabalan 1998).

Sucrose plays a key role in flower morphogenesis. The flowers formed in this experiment could not develop further until the shoots were transferred to medium having higher concentration of sucrose. Medium without sucrose did not induce flowering and the concentration of sucrose (3%) induce flowering and the percent of flowering and fruiting shoots tended to increase. The important role of sugar in floral induction under *in vitro* conditions has been observed in many species (Franklin et al. 2000; Yadav and Singh, 2011; Cha-um et al. 2012). Vu et al. (2006) suggested that sucrose was the only factor needed for the induction of floral buds or initial development while other factors are important in later stages of *in vitro* floral development.

Table 2. Effect of different concentrations of Kn, BAP, NAA and IAA on *in vitro* flowering from nodal explants of *O. corniculata*

Plant growth regulators (mg/l)				Sucrose concentration (%)	Explant	Number of flowers/culture
Kn	BAP	NAA	IAA			
1.0	-	0.5	-	3	Axillary bud	1
2.0	-	0.5	-	3	Axillary bud	3
3.0	-	0.5	-	3	Axillary bud	2
-	1.0	0.5	-	3	Axillary bud	-
-	2.0	0.5	-	3	Axillary bud	-
-	3.0	0.5	-	3	Axillary bud	-
1.0	-	-	0.5	3	Axillary bud	1
2.0	-	-	0.5	3	Axillary bud	2
3.0	-	-	0.5	3	Axillary bud	2
-	1.0	-	0.5	3	Axillary bud	-
-	2.0	-	0.5	3	Axillary bud	-
-	3.0	-	0.5	3	Axillary bud	-

Observation: After 8 weeks, **-** =No flowering.

Although strength of MS medium appeared to be an important factor in determining rooting efficiency but in our experiment when all the other parameters were kept at the optimum level, the decrease or increase in it also played important role in inducing flowering and fruiting. Out of various strengths of MS salts used, medium with half strength of MS salt was found best for *in vitro* flowering as well as fruiting (Table 2).

Half strength MS medium has been effective for inducing *in vitro* flowering in many other plants like *Rotula aquatic* **(**Chithra et al. 2004**)**, *Capsicum annuum* **(**Bodhipadma and Leung 2003**)** and *Melia azedarach* **(**Thakur et al. 1998**)**. The reason behind this may be reduction in nitrogen which forms the major part of inorganic and organic salts of MS medium and according to the floral nutrient diversion hypothesis increase in C/N ratio increases the flower bud induction **(**Ziv and Naor, 2006**).**

Light is a very important factor for induction and development of flowers under *in vitro* conditions. Cultures maintained under high light intensity (40-50 µmol m^{-2} s^{-1} PFD) produced flowers that did not bloom and these cultures did not produce fruits or if fruits were produced they did not mature. However, under low light (10-15 µmol m^{-2} s^{-1} PFD) conditions, flowers (Fig. 1G & H), as well as fruits (Fig. 1I), developed properly. Low light conditions were also found to be effective for flower induction in *Crocus sativus* (Jun et al. 2007). The shoots did not flower in the dark.

All the flowers produced *in vitro* were bisexual and contained all floral parts (Fig. 1J) but these flowers were smaller in size than the flowers of plants grown in field conditions (Fig. 1K). These *in vitro* developed flowers self-fertilized and formed fruits. All the mature fruits were also smaller in size and contained a lesser amount of seeds than the *in vivo* (Fig. 1L & M).

The seeds (resembling the normal seeds in size and colour) collected from fully matured fruit shows 75% and 60% viability under *in vitro* (Fig.1N) and *in vivo* conditions respectively.

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