African Journal of Biotechnology Vol. 11(31), pp. 7906-7911, 17 April, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.4239
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Plant regeneration and stimulation of *in vitro* flowering in *Eruca sativa* Mill.

Madan Mohan Sharma*, Mukta Dhingra, Anju Dave and Amla Batra

Department of Botany, University of Rajasthan, Jaipur, India.

Accepted 27 February, 2012

Explants such as apical buds, axillary buds, cotyledons, cotyledonary nodes, leaves, hypocotyls and immature embryonal axes from *in vitro*-grown plantlets were inoculated on the Murashige and Skoog (MS) medium supplemented with 4.44 µM 6-benzylaminopurine in combination with 2.85 µM indole-3-acetic acid. Best multiple shoots formation was obtained with cotyledonary nodes. Each inoculated explant produced 18.10 ± 0.66 shoots within 2 to 3 weeks. These shoots were separated carefully and were transferred to the fresh half strength MS solid medium with indole-3-butyric acid (4.90 µM) for the development of the roots. These *in vitro*-developed plantlets produced flowers on the same medium with supplementation of 6-furfuril kinetin (0.23 µM). These plantlets were successfully transferred to the soil where they grew well for 8 to 10 weeks with 80% survivability.

Key words: *Eruca sativa*, cotyledonary nodes, *in vitro* regeneration, *in vitro* flowering, shoot multiplication.

INTRODUCTION

Eruca sativa Mill. belonging to the family Brassicaceae attain a height of 2 to 4 feet, it is branched, erect, hairy, and leaves are borne on the stem in an alternate fashion. Flowers are self sterile bright yellow in colour. Fruit is siliqua, one inch in length. Seeds are small, light reddish brown in colour. Flowering occurs in the month of January to April. Effect of high temperature of Rajasthan is very much pronounced on flowering and consequently on seeds. A high temperature during vegetative growth produces the highest seed yield. High temperature during early seed formation affects the seed oil content and quality adversely. The plant is able to withstand temperature up to 40°C. It has photoperiod ranges of 10 h. Maturity of the plant takes place from 60 to 340 days. Brassicaceae family contributes fifth major portion amongst the oil seed crops. It also has diversified medicinal and therapeutic properties like astringent, diuretic, digestive, emollient, tonic, depurative, laxative, rubefacient, stimulant, anti-tumor, anti-ulcer and hepato-protective activities (Yaniv et al., 1998; Modlinger et al., 2004). It is mainly used for the production of oil, which is being used for domestic lighting. It is also used in industries. Industrial uses of high erueic oil content could be expanded since long chain fatty acids involved have considerable advantages. Oil seeds constitute important group of crops as they are an easily available source of energy and nutrition. Inspite of the recalcitrant nature of certain oil seed crops like cumin, sesamum, arachin, Jatropha, Ricinus, Brassica, etc., reports are available on their *in vitro* regeneration (Batra and Dhingra, 1991; Khan et al., 2009).

Plant regeneration of *E. sativa* via shoot tip culture, somatic embryogenesis, mesophyll protoplasts, zygotic embryos and isolated microspore culture has previously been reported (Ahloowalia, 1987; Sikdar et al., 1987; John and Batra, 1994; Zhang et al., 2005; Leskovsek et al., 2008), but the regeneration frequencies were low and the protocols were complicated.

This paper presents the findings of an experiment to work out a suitable protocol on efficient and reliable regeneration system via cotyledonary nodes in *E. sativa*

*Corresponding author. E-mail: drmadansharma@gmail.com

Abbreviations: BAP, 6-Benzylaminopurine; **Kn,** 6-furfuryl amino purine; **IAA,** indole-3- acetic acid; **IBA,** indole-3-butyric acid; **NAA,** α-naphthalene acetic acid.

and the role of phytohormones in *in vitro* flowering in this plant species. Thus, the protocol developed during the present investigation may contribute towards improvement of this crop quality in Brassicaceae family.

MATERIALS AND METHODS

Procurement of plant materials

Mature dried seeds of *E. sativa* were procured from Durgapura Agriculture Research Station, Jaipur (Rajasthan) India. Seeds were washed three to four times with distilled water. Seeds were then treated with 0.1% (v/v) Tween-20 solution for 10 min, rinsed with distilled water at least thrice. Further surface-sterilization treatment was conducted in laminar air flow chamber. Seeds were dipped in 0.1% (w/v) freshly prepared aqueous mercuric chloride ($HgCl_2$) solution for 3 to 5 min, followed by 30 s in 70% (v/v) ethanol, and then washed three to four times with sterile distilled water. Surface sterilized seeds were inoculated on half strength MS medium supplemented with 1.0% sucrose and 0.8% agar.

Culture conditions

All the culture were incubated at 25 ± 2°C with 55 to 65% relative humidity under a 16 h light/8 h dark cycle at the light intensity of 40 µ mol m^{-2} s^{-1} provided by cool and white fluorescent tubes. The *in vitro* germinated seedlings were used as stock mother plant. Cotyledonary node segments excised from two weeks old seedlings served as explants and cultured on MS medium (Murashige and Skoog 1962), supplemented with BAP (1.1 to 22.19 µM) and Kn (1.16 to 23.23 µM) alone or in combination with different concentrations of IAA (0.57 to 14.27 µM), IBA (0.49 to 12.26 µM), NAA (0.54 to 13.43 µM) and 2,4-D (0.45 to 11.31 µM). MS medium was supplemented with 3% sucrose. The pH of the medium was adjusted to 5.8 with 0.1 N NaOH or 0.1 N HCl before adding 0.8% agar (bacteriological grade, Himedia) and autoclaved. The effects of orientation of the explant were also studied. Cotyledonary nodes were placed horizontally as well as vertically on MS medium. The cultures were transferred to fresh medium at three weeks intervals.

In vitro rooting and flowering

Well developed elongated shoots (5.5 to 7.5 cm) with four to five leaves regenerated on MS medium supplemented with BAP and IAA were excised and transferred to solidified half-strength MS medium containing 3% sucrose and different concentrations of NAA (0.54 to 13.43 µM), IAA (0.57 to 14.27 µM) and IBA (0.49 to 12.26 µM) alone or in combination with cytokinin (BAP and kinetin) for root and flower induction. *In vitro* separated shoots were also sub cultured on hormone free MS medium as control for the root induction. The number of flowers per shoot was counted.

Hardening and acclimatization of plants

Rooted plantlets were taken out from culture vials and washed with distilled water delicately to remove adhered agar and transferred to earthen pots containing autoclaved soil and vermicompost (3:1). The pots were covered with inverted glass beakers to maintain high humidity. Plants were provided with MS salts as few drops gradually on alternate day along with distilled water. The percentage of explants forming shoots was regularly recorded and readings were taken after 3 weeks of culture. The mean number of shoots per explant was determined after 14 and 21 days of culture. All experiments were repeated at least thrice and consisted of 28 replicates. Data were analyzed statistically.

RESULTS AND DISCUSSION

Multiple shoot induction

During the present studies, micropropagation was obtained by *in vitro* grown explants. Two weeks old germinated seedlings were used for the excision of explants (Figure 1A). However, for these explants, surface sterilization was not required since they were already grown under aseptic conditions. Explants like apical bud, axillary bud, cotyledons, cotyledonary nodes, leaves, hypocotyls, immature embryonal axes, etc. were inoculated on nutrient MS medium along with 0.8% agar, 3.0% sucrose and pH 5.8 under aseptic conditions. Protocol for optimum regeneration was developed via the culture of cotyledonary nodes.

However, reports are available on regeneration of plantlets via cotyledonary node in a variety of plants like *Psoralea corylifolia* L., *Aegle marmelos* (L.) Corr., *Vigna mungo* (L.) Hepper, *Vigna radiata* L., *Pterocarpus marsupium* Roxb. (Jeyakumar and Jayabalan, 2002; Anis et al., 2005; Muruganantham et al., 2005; Sita Mahalakshmi et al., 2006; Nayak et al., 2007).

Cotyledonary node segments inoculated on MS medium augmented with BAP (1.1 µM) gave elongated shoots but it did not multiply the number of shoots till two weeks (Figure 1B and Table 1). Further, multiple shoot proliferation was obtained after 15 days of cotyledonary nodes subcultured on MS medium supplemented with BAP (4.44 µM) and IAA (2.85 µM). Cytokinin alone did not show significant results. However, IAA in combination with BAP produced the optimal number of shoots (18.10 ± 0.66) (Table 2). Horizontally inoculated explants showed callusing at the cut ends and gave only 2- to 3 shoots, while vertically placed explants gave rise to shoot clusters and callus from the base of the explants which was embedded inside the medium (Figure 1C). Except IAA, none of the auxins was found to accelerate the rate of multiplication (data not shown). These shoots further increased in number and length (5.5 to 7.5 cm.) within 4 weeks, when they were separated carefully and sub-cultured onto MS medium with same hormonal regimes (Figures 1D, 2A and Table 2).

In vitro rooting

These *in vitro* developed shoots were subjected to rooting media regimes. Rooting was scanned by incorporating each auxin such as IAA (0.57 to 14.27 µM), IBA (0.49 to 12.26 µM) and NAA (0.54 to 13.43 µM)

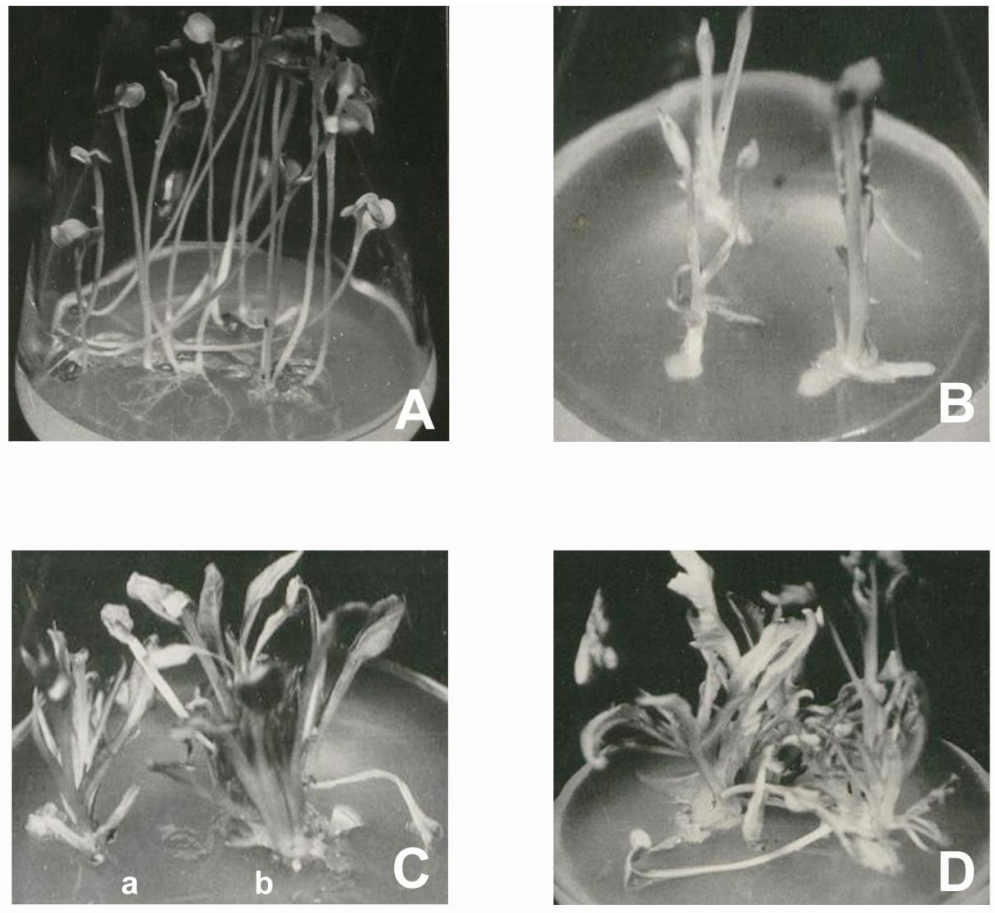

Figure 1. *In vitro* seed germination and further multiplication of shoots through cotyledonary node culture. A, Two weeks old sterile seedlings of *E. sativa* on ½ MS medium; B, Elongation of a single shoot at two weeks on MS + BAP (1.1 µM); C, Shoot multiplication at two weeks on MS + BAP (4.44 µM) + IAA (2.85 µM); a, Horizontally placed explant; b, vertically placed explant; D, Shoot clusters at four weeks on the same medium.

Table 1. Effect of cytokinin on shoot proliferation from the cotyledonary node explant of *Eruca sativa* Mill. on the MS medium.

Plant growth regulator and concentration (µM)	Percentage explants showing shoot proliferation	Mean number of shoots produced/explant
BAP (1.1)	40[z]	1.25±0.88[y]
BAP (2.22)	70	6.55±0.25
BAP (4.44)	85	9.60 ± 4.50
BAP (8.87)	60	4.66 ± 0.21
BAP (17.74)	50	3.70± 0.60
BAP (22.19)	30	0.66 ± 0.21
Kn (1.16)	35	1.00 ± 0.25
Kn (2.32)	50	1.33 ± 0.21
Kn (4.65)	40	1.16 ± 0.17
Kn (9.29)	30	0.50 ± 0.22
Kn (18.58)	25	0.33 ± 0.21
Kn (23.23)	25	0.20 ± 0.16

[z]Values represent mean ± SE of eight replicates per treatment. [y]Mean values within the column followed by the same letter are not significantly different by the Tukey's test at 0.05% probability level.

Table 2. Effect of IAA with optimal concentration of BAP on shoot proliferation from cotyledonary nodes of *Eruca sativa* Mill. in MS medium.

Growth regulator	Percentage explants showing shoot proliferation	Mean number of shoots produced/explant
BAP (4.44 µM) + IAA (0.57 µM)	60	12.81±1.80
BAP (4.44 µM) + IAA (1.425 µM)	70	13.93±0.91
BAP (4.44 µM) + IAA (2.85 µM)	80	18.10±0.66
BAP (4.44 µM) + IAA (5.71 µM)	50	11.22±0.44
BAP (4.44 µM) + IAA (11.42 µM)	30	7.4 ± 0.03
BAP (4.44 µM) + IAA (14.27 µM)	30	7.25±0.478

Values represent mean ± SE of eight replicates per treatment. Mean values within the column followed by the same letter are not significantly different by the Tukey's test at 0.05% probability level.

separately and in combination with BAP (1.1 to 22.19 µM) and Kn (1.16 to 23.23 µM) in half strength MS medium. On hormone free MS basal medium, no rhizogenesis response was observed, leaves turned yellow and the shoots became necrotic (Figure 2B). IAA did not induce rooting (data not shown). NAA at concentration as low as 2.69 µM produced profuse fibrous roots, while slightly higher concentration (5.37 µM) induced root formation accompanied by callusing (Figure 2C). Best response at 4.90 µM of IBA and Kn (0.23 µM) was 60 to 80% rooting which was obtained (Figure 2D).

Effect of phytohormones on flower induction

In vitro rooting preceded flowering in all the responding shoots. Shoots without roots did not produce flowers. Flowers were initiated in the media containing auxin (IBA) and cytokinin (BAP and kinetin) in combination. Thus, it is evident that the presence of auxin and cytokinin is essentially required for the induction of flowering in *E. sativa in vitro,* as no inflorescences were observed in the control explants devoid of cytokinins. The number of flowers was influenced by both the type of cytokinin (BAP and Kn) added to the media and the concentration thereof (Figure 2E, Grapg-1 and 2). Among the auxins tested, IBA (4.90 µM) was more efficient than NAA and IAA in induction and maturation of *in vitro* flowers in combination with Kn.

Cytokinins are constituents of the floral stimulus transported in phloem sap to the apex in response to a photoperiodic treatment inducing flowering (Bernier et al., 1993). The role of cytokinin in floral morphogenesis was also reported by various scientists (Taylor et al., 2005; Verma and Singh, 2007; Sudarshana et al., 2008). However, Sharma et al. (2011) also reported the *in vitro* flowering in *Portulaca oleracea* L. on Kn along with gibberellic acid, which was also in favour of the present results. In contrast to the above results, BAP (13.3 mM) in combination with Kn (9.3 mM) stimulated flowering in *Rosa hybrida* cv. 'Heirloom under photoperiod of 12/12 (light/dark cycle) (Kanchanapoom et al., 2010).

Effect of sucrose and ammonium nitrate on flower induction

Different concentrations of sucrose (0.0 to 6.0%) were tested, out of all, 3% sucrose induced rooting and flowering. Maximum number of flowers were induced on half strength ammonium nitrate (NH_4NO_3) (0.825 g l^{-1}), when compared with full strength (1.65 g l^{-l}) ammonium nitrate in the medium. Similarly, Franklin et al. (2000) also reported *in vitro* flowering in *Pisum sativum* L. using half strength ammonium nitrate in the medium. In contrast with the present results, Chen and Li (1993) reported successful *in vitro* flower induction in tobacco using free amino acids.

Effect of age of cultures on flower induction

The initiation of flowering *in vitro* depends on the age of the cultures derived *in vitro*. Plantlets derived from *in vitro* primary cultures showed maximum number of flowers as compared to the cultures with increased age. However, Wang et al. (2002) reported the induction of *in vitro* flower buds in rose after 15 days of subculturing of the shoots.

Hardening and acclimatization

These rooted plantlets were carefully taken out from the agar media without causing damage to the delicate root system. Roots were then thoroughly washed with sterile distilled water to remove even the traces of adhering agar of the media. These recreated plantlets were then transferred to plastic pots having a mixture of vermi-compost and autoclaved soil (1:3). These were kept in mist chamber for first two weeks with high humidity,

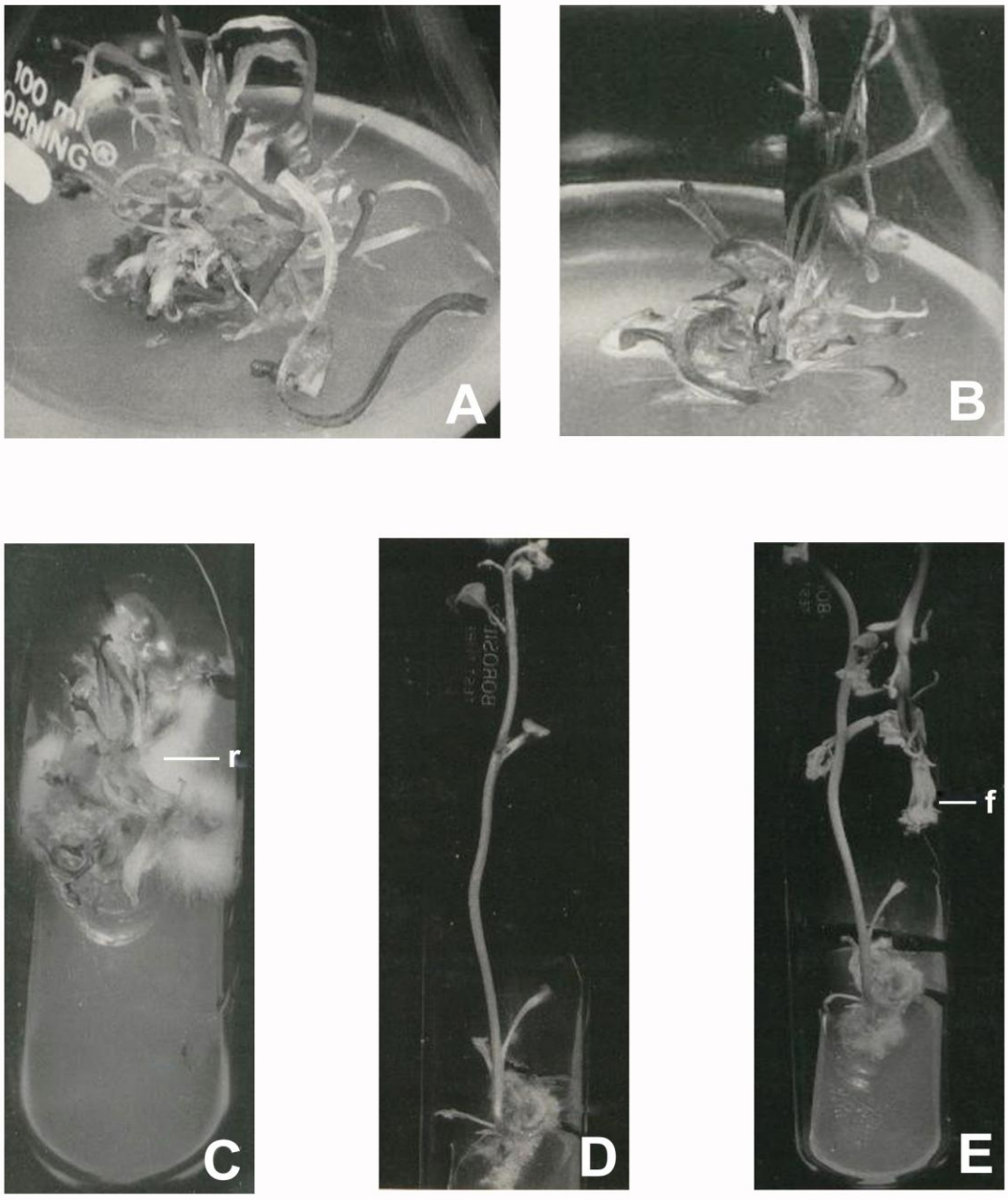

Figure 2. Platelet formation in *E. sativa*. A, Shoot proliferation on MS + BAP (4.44 µM) + IAA (2.85 µM); B, Separated shoots on hormone free MS medium; C, Rooting from isolated shoots on MS + NAA (2.69 µM); D, Rooting and shoot elongation in single shoot on ½ MS + IBA (4.90 µM); E, Flower and bud induction on MS + Kn (0.23 µM) + IBA (4.90 µM); f, Flower bud; r, roots.

controlled temperature and light. After two weeks, the plants were gradually exposed to natural conditions for a few hours and then this duration of exposure was increased every day from 21st till 28th days, respectively. Similar procedure of hardening was also reported by many earlier scientist in different plant species such as *Curculigo orchioides* Gaertn. (Bhavisha and Jasrai, 2003) and *Phoenix dactylifera* (Bekheet et al., 2007). In contrast to this, other scientists adopted different method and potting mixture (cocopeat and soilrite) for hardening of the plantlets (Kavyashree, 2007; Girijashankar, 2011).

Conclusions

In conclusion, we reported an *in vitro* regeneration and

flowering in aseptic conditions of *E. sativa*. The flowers were bisexual and looked like general flowers. In view of these, the present protocol provides a useful system in plant breeding and crop improvement. It can also be used for the study of physiological signals that induce *in vitro* flowering.

REFERENCES

Ahloowalia BS (1987). Somatic embryogenesis and plant regeneration in *Eruca sativa*. Crop Sci. 27: 813-814.

Anis M, Mohd KH, Shahzad A (2005). *In vitro* plantlet regeneration of *Pterocarpus marsupium* Roxb., an endangered leguminous tree. Curr. Sci. 88(6): 861-863.

Batra A, Dhingra M (1991). Production of plantlets of *Eruca sativa in vitro*. J. Phytol. Res. 4(1): 73-77.

Bekheet SA, Taha HS, Saker MM, Solliman ME (2007). Application of Cryopreservation technique for *in vitro* grown Date Plam (*Phoenix dactylifera* L.) cultures. J. Appl. Sci. Res. 3(9): 859-866.

Bernier G, Havelange A, Houssa C, Petitjean A, Lejeune P (1993). Physiological signals that induce flowering. Plant Cell, 5: 1147-1155.

Bhavisha BW, Jasrai YT (2003). Micropropagation of an Endangered Medicinal Plant: *Curculigo orchioides* Gaertn. Plant Tissue Cult. 13(1): 13-19.

Chen Y, Li W (1993). Floral gradient in flowering tobacco in relation to free amino acids. Cell Res. 3: 85-92.

Franklin G, Pius PK, Ignacimuthu S (2000). Factors affecting *in vitro* flowering and fruiting of green pea (*Pisum sativum* L.). Euphytica, 115: 65-73.

Girijashankar V (2011). Micropropagation of multipurpose medicinal tree *Acacia auriculiformis*. J. Med. Plants Res. 5(3): 462-466.

Jeyakumar M, Jayabalan N (2002). *In vitro* plant regeneration from cotyledonary node of *Psoralea corylifolia* L. Plant Tissue Cult. 12(2): 125-129.

John N, Batra A (1994). Plantlet formation from shoot tip culture of *Eruca sativa* Mill. J. Ind. Bot. Soc. 73(3-4): 367-368.

Kanchanapooma K, Sakpetha P, Kanchanapoomb K (2010). *In vitro* flowering of shoots regenerated from cultured nodal explants of *Rosa hybrida* cv. 'Heirloom'. Sci. Asia, 36: 161-164.

Kavyashree R (2007). A repeatable protocol for *in vitro* micropropagation of mulberry variety S54. Ind. J. Biotech. 6: 385-388.

Khan MMA, Hassan I, Ahmad SD, Shah SH, Batool F (2009). *In vitro* regeneration potentiality of oil seed *Brassica* genotypes with differential BAP concentration. Pak. J. Bot. 41(3): 1233-1239.

Leskovsek L, Jakse M, Bohanec B (2008). Doubled haploid production in rocket (*Eruca sativa* Mill.) through isolated microspore culture. Plant Cell Tissue Organ Cult. 93:181-189.

Modlinger PS, Wilcox CS, Aslam S (2004). Nitric oxide, oxidative stress, and progression of chronic renal failure. Semin. Nephrol. 24: 354-365

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiol. Plant 15: 473-497.

Muruganantham M, Ganapathi A, Amutha S, Vengadesan G, Selvaraj N (2005). Shoot regeneration from immature cotyledonary nodes in black gram [*Vigna mungo* (L.) Hepper]. Ind. J. Biotech. 4: 551-555.

Nayak P, Behera PR, Manikkannan, T (2007). High frequency plantlet regeneration from cotyledonary node cultures of *Aegle marmelos* (L.) Corr. *In Vitro* Cell. Dev. Biol. Plant, 43: 231-236.

Sharma MM, Singh A, Verma RN, Ali DZ, Batra A (2011). Influence of PGRS for the *in vitro* plant regeneration and flowering in *Portulaca oleracia* l.: A medicinal and ornamental plant. Int. J. Bot. 7(1): 103-107.

Sikdar SR, Chatterjee G, Das S, Sen SK (1987). Regeneration of plants from mesophyll protoplasts of the wild crucifer *Eruca sativa* Lam. Plant Cell Rep. 6: 486-489.

Sita Mahalakshmi L, Leela T, Kiran B, Naresh B, Devi P (2006). *In vitro* plant regeneration from the petioles of primary leaves of Mungbean *Vigna radiata* L. Plant Biotech. 23: 409-411.

Sudarshana MS, Niranjan MH, Girisha ST (2008). *In vitro* flowering, somatic embryogenesis and regeneration in *Boerhaavia diffusa Linn*. A medicinal plant. Global J. Biotech. Biochem. 3(2): 83-86.

Taylor NJ, Light ME, Van Staden J (2005). *In vitro* flowering of *Kniphofia leucocephala*:influence of cytokinins. Plant Cell Tissue Org. Cult. 83: 327-333.

Verma R, Singh RR (2007). Regeneration and *in vitro* flowering in *Brassica campestris* (L.) Var. Bhavani. Our Nature, 5: 21-24.

Wang GY, Yuan MF, Hong Y (2002). *In vitro* flower induction in roses. *In Vitro* Cell. Dev. Biol. Plant 38: 513-518.

Yaniv Z, Schafferman D, Amar Z (1998). Tradition, Uses, and Biodiversity of Rocket (*Eruca sativa*) in Israel. Econ. Bot. 52: 394-400.

Zhang T, Cao ZY, Wang XY (2005). Induction of somatic embryogenesis and plant regeneration from cotyledon and hypocotyl explants of *Eruca sativa* Mill. *In Vitro* Cell. Dev. Biol. Plant, 41: 655-657.