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.

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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 silique, 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 hepatoprotective 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 (Ahlouwalia, 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*.
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 (HgCl₂) 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 µmol 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 Jayabal, 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 subcultured 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.

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

<sup>z</sup>Values represent mean ± SE of eight replicates per treatment. <sup>y</sup>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, Grap-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 (NH₄NO₃) (0.825 g l⁻¹), when compared with full strength (1.65 g l⁻¹) 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 vermicompost and autoclaved soil (1:3). These were kept in mist chamber for first two weeks with high humidity,

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

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

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.
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**


