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Somatic embryogenesis and plant regeneration from leaf explants of *Rumex vesicarius* L.

Lavanya Kakarla, Chakravarthy Rama*, Mahendran Botlagunta, Krishna M. S. R and Pardha Saradhi Mathi

Department of Biotechnology, K L University (Koneru Lakshmaiah Education Foundation) Vaddeswaram, Guntur 522502, Andhra Pradesh, India.

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An attempt was made to study the somatic embryogenesis and plant regeneration from the *in vitro* leaf explants of *Rumex vesicarius* L. a renowned medicinal plant, which belongs to polygonaceae family. Effective *in vitro* regeneration of *R. vesicarius* was achieved via young leaf derived somatic embryo cultures. Embryogenic callus was induced from leaf explants on Schenk and Hildebrandt (SH) medium supplemented with various concentrations of 2,4-dichlorophenoxy acetic acid (2,4-D) (0.5 to 3.0 mg/l) along with Kinetin (Kn) (0.5 mg/l). High frequency of somatic embryogenesis was effective on SH medium with 2, 4-D (2.5 mg/l) + Kn (0.5 mg/l) from leaf explants. Secondary somatic embryogenesis was also observed when primary somatic embryos were subculture on the same somatic embryo induction medium. Well developed cotyledonary shaped embryos regenerate 80% of shoots on media containing 2,4-D 0.5 mg/l + 2.0 mg/l BA. The regenerated shoots transferred to rooting media containing Indole- 3-butyric acid (IBA). Efficient rooting of 90% was noted on SH media with 1.0 mg/l IBA. Finally, these *in vitro* regenerated plantlets were hardened, acclimatized and successfully transferred to the field. The post transplantation survival rate of these regenerated plants was 65 to 70%. The *in vitro* regenerated plants and flowers were similar to mother plants. This protocol will be useful for genetic transformation experiments in *R. vesicarius* L.

Key words: *Rumex vesicarius* L, 2,4-dichlorophenoxy acetic acid (2,4-D), kinetin (Kn), Benzyl adenine (BA), Indole- 3- butyric acid (IBA).

INTRODUCTION

Somatic embryogenesis (SE) is the ultimate developmental pathway by which somatic cells develop into structures that resemble zygotic embryos (that is, bipolar and without vascular connection to the parental tissue) through an orderly series of characteristic embryological stages without fusion of gametes (Jimenez, 2005). SE has been traditionally divided into two main stages, namely induction and expression. In the former, somatic cells acquire embryogenic characteristics by means of gene expression (Feher et al., 2002). The ontogeny,

*Corresponding author. E-mail: drrchakravarthy@gmail.com. Tel: 09912494464.

physiological, biochemical and media properties are required for somatic embryogenesis (Victor, 2001). Typical globular, heart, torpedo and cotyledonary stages of somatic embryos are different from various kinds of explants. True-to–type nature of the somatic embryo derived plantlets has been reported (Tokuhara and Mii, 2001). As a result any plant which needs to be altered by genetic engineering and transgenesis requires a pre developed protocol for successful regeneration through somatic embryogenesis (Birch, 1997; Thangavel et al., 2014). Developing a protocol for plant regeneration through somatic embryogenesis will immensely benefit the plant conservation programs too as it is a shorter and viable method for producing a large number of plantlets.

The genus *Rumex* belongs to family Polygonaceae that comprises about 150 species widely distributed around the World. The main chemical constituents of *Rumex* are anthraquinones and flavanoids (Cunningham, 1993). *Rumex vesicarius* L. (English: bladder dock, Hindi: Chooka, Sanskrit: Amlavetasa, Telugu: Chukkakura) a common green leafy vegetable is also used in herbal and ayurvedic formulations. It is a branched succulent herb and is distributed throughout India (Alam, 2012). The plant extract have been used to reduce cholesterol levels, biliary disorders (Rechinger, 1984; Mona et al., 2013) and also it showed significant effect on antioxidant (Palani and Ramakrishnan, 2011; 2012; Sakkir et al., 2012) and antimicrobial activities (Al Akeel et al., 2014; Ramesh and Asha, 2013).

In vitro regeneration of *R. vesicarius* L. has been achieved by researchers using explants like shoot tips, nodal explants, leaves and callus (Panduraju et al., 2009; Abo El-soud et al., 2012; Nandini et al., 2013; Lavanya et al., 2013). However, there is no report on the induction of somatic embryogenesis. This is an alternative method for plant propagation over regeneration via organogenesis. The plants regenerate *via* somatic embryogenesis is of single cell origin with true-to-type and are produced in large numbers within a short period (Ammirato et al., 1983; Lavanya et al., 2014).

Many researchers have emphasized that somatic embryogenesis is preferred method for rapid *in vitro* multiplication of plants (Moon et al., 2013), production of artificially synthetic seeds (Ravi and Anand, 2012), Agro bacterium mediated genetic transformation studies and regeneration of transgenic plants (Satyanarayana et al., 2012). In the present study we made an attempt to establish a reliable and efficient protocol for the induction of somatic embryogenesis and plant regeneration from leaf explants of *R. vesicarius* L.

MATERIALS AND METHODS

Culture medium and conditions

The seeds of *R. vesicarius* were collected from the plants grown in the research field of Department of Biotechnology, K L University. They were soaked in sterile distilled water for 24 h, later cleaned with 5% tween-20 (w/v) and thoroughly washed in running tap water 3 to 4 times. Subsequently, they were surface sterilized with 0.1% w/v $HgCl_2$ for 2 to 3 min followed by rinsing with sterile distilled water for 2 to 3 times and germinated aseptically on SH medium (Schenk and Hildebrandt, 1972). Finally, these seeds were flame sterilized with Whatman filter paper and supplemented on the surface of the nutrient culture medium SH without growth regulators. Effective plantlets developed from these seeds within one week. After two weeks, leaves were taken as explants for callus induction. We found that compared to *ex vitro,* the *in vitro* leaf explants was found to be appropriate as it was responding well under *in vitro* conditions.

Embryo germination and plantlet formation

For germination and plantlet formation cotyledonary stage somatic embryos were transferred to SH medium supplemented with 0.5 mg/l 2,4-D + 0.5 to 3.0 mg/l BA.

Culture conditions

SH media were supplemented with 3% (w/v) sucrose and solidified with 0.8% (w/v) agar (Himedia). After adding all the growth regulators, the pH of the medium was adjusted to 5.6 with 1 N NaOH or 1 N HCL and autoclaved at 121°C with 15 p.s.i pressure for 15-20 min. All the cultures were incubated at 25±2°C under a 16 h photoperiod. Light intensity of 40 to 50 $\mu molm^{-2}s^{-1}$ was provided by using cool white fluorescent tubes. The cultures were transferred to fresh medium after an interval of four weeks. For each hormonal treatment 20 replicates were raised and the experiments repeated at least twice. Data on somatic embryogenesis and germination were statistically analyzed using Turkey's HSD test at p=0.05 with SPSS ver.13.0. The results are expressed as Mean ± SE of two experiments.

Acclimatization

The plants were taken out from the cultured tubes and washed with sterile distilled water under aseptic conditions to remove agar medium. They were shifted to plastic pots containing sterile vermiculate: soil (1:1), covered with polythene bags in order to maintain 80 to 85% relative humidity and kept in culture room for 3 weeks. Later, they were transferred to earthen pots containing garden soil and maintained in the research field.

RESULTS AND DISCUSSION

The *in vitro* leaf explants were spliced at the terminal ends using scalpels and inoculated on SH medium containing different concentrations of 2,4-D (0.5 to 3.0 mg/l) in combination with Kn (0.5 mg/l). Highly differentiated, friable callus was induced from these explants in one week (Figure 1a). Within 10 to 15 days of culture inoculation greenish friable callus was observed (Figure 1b). Green nodular embryogenic callus was noticed after three weeks of culture inoculation from these explants (Figure 1c). When the explants of embryogenic callus was cut into fragments and cultured on the same induction medium for an extended period of three to four months, secondary somatic embryos with different shapes such as globular, heart, torpedo and cotyledonary

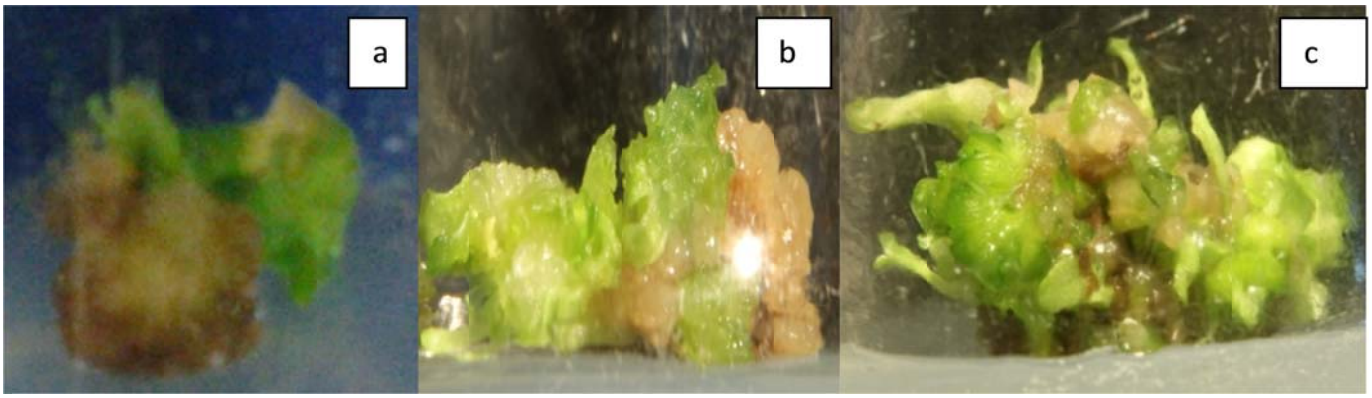

Figure 1. *In vitro* regeneration *via* Somatic embryogenesis in leaf explant cultures of *Rumex vesicarius* L. **a).** Initiation of callus from leaf explant. **(b).** Profuse, greenish and friable callus formed from leaf explants. **(c).** A type of embryogenic callus after 3 weeks in culture, the callus was green and nodular with the presence of abundant somatic embryos.

Figure 2. *In vitro* regeneration *via* somatic embryogenesis in leaf explant cultures of *Rumex vesicarius* L. showing somatic embryogenesis in *Rumex vesicarius* L. **1.** Initiation of spherical shaped globular embryoids from leaf explants in *R. vesicarius* L. **2.** Transformation of globular embryoids into heart shaped embryo. **3.** Modification of heart shaped embryo into torpedo shaped embryonic form. **4.** Development of cotyledonary shaped embryonic buds. **5.** Nodule, shoot buds regeneration from cotyledonary buds.

embryoids were observed after four to six weeks of culture inoculation (Figure 2).

Somatic embryos proliferation occurred in two ways such as direct somatic embryos formation from explants and indirect from repetitive organogenesis. SH medium with 0.5 mg/l 2,4-D + 0.5 mg/l Kn showed 30% of somatic embryoids induction. At 1.0 mg/l 2,4-D + 0.5 mg/l Kn 45% of embryoids were observed. 60% of somatic embryoids

Table 1. Effect of various concentrations of 2,4-D and 0.5 mg/l Kn on Somatic embryogenesis in leaf explants of *R. vesicarius* L.

Growth regulators mg/l 2,4-D+Kn	Percentage of response for somatic embryo formation	Average number of somatic embryos/explants (Mean ± SE)
0.5+0.5	30	8.66^{A} ± 0.930
1.0+0.5	45	9.44^{A} ± 1.125
1.5+0.5	60	16.33^{B} ± 1.873
2.0+0.5	75	24.20^{B} ± 2.464
2.5+0.5	85	36.0^{C} ± 3.120
3.0+0.5	65	18.46^{B} ± 2.036

Table 2. Effect of 2,4-D and BA on germination of shoots from somatic embryos in *R. vesicarius* L.

Growth regulators mg/l 2,4-D+BA	Percentage of somatic embryo germination	Average number of shoots from somatic embryos (Mean ± SE)
0.5+0.5	40	5.50^{A} ± 0.62
0.5+1.0	65	9.23^{B} ± 1.04
0.5+2.0	80	16.875^{C} ± 1.90
0.5+3.0	55	7.63^{B} ± 0.899

was observed at 1.5 mg/l 2,4-D + 0.5 mg/l Kn. Among the various concentrations of 2,4-D tested in combination with 0.5 mg/L Kn, the percentage of explants responded for somatic embryo formation was found to be higher at 2.5 mg/l 2,4-D + 0.5 mg/l Kn in leaf explants with maximum of 36.0 ± 3.12 somatic embryo production. However, at the concentration of 2,4-D higher than 2.5 mg/l the percentage of somatic embryo induction was lower (Table 1). The development of somatic embryos was asynchronous. As a result, various stages of embryo development could be observed in the same cluster of embryos originally from the explants. When these embryos with different developmental stages were transferred to the same medium, further germination in them was not observed.

Embryo germination and plantlet formation

The cotyledonary embryos proliferated to nodular buds with synthesis of shoot bud initiation effectively on 2,4-D 0.5 mg/l + 2.0 mg/l BA when compared to other hormonal concentrations. Highest shoot bud initiation was found to be 80% at 2,4-D 0.5 mg/l + 2.0 mg/l BA, 65% of germination was observed in 2,4-D 0.5 mg/l + 1.0 mg/l BA, 55% at 2,4-D 0.5 mg/l + 3.0 mg/l BA and 40% at 2,4-D 0.5 mg/l + 3.0 mg/l BA. Hence, it was observed that the increase in the growth hormone concentration showed gradual decrease in germination of shoots. Therefore, SH medium with 2,4-D 0.5 mg/l + 2.0 mg/l BA is proven to be effective for germination of maximum number of shoots 16.875 ± 1.90 from cotyledonary embryoids in *R. vesicarius* (Table 2, Figure 3a and b). Later, the *in vitro* regenerated shoots were separated from the embryogenic callus and sub cultured on to fresh media containing 2.0 mg/l BA. These plantlets elongated and produced multiple shoots within two weeks (Figure 4a and b).

After elongation, the *in vitro* regenerated shoots were transferred onto rooting media containing IBA (0.5 to 2.0 mg/L). The highest rooting (90%) was noted on SH medium containing 1.0 mg/L IBA with average number of roots (6.38 ± 0.687) (Table 3, Figure 4c). Increasing or decreasing the concentrations of IBA resulted in lower rooting. Later, these *in vitro* regenerated plantlets were transferred to plastic pots containing sterile vermiculite: soil (1:1) mixture. Finally, they were shifted to earthen pots after hardening in the culture room and maintained in the research field under shady conditions. The survival percentage of plants was found to be 70 to 80%. The plants were normal; morphological and floral characters were found to be similar to the donor plants (Figure 4d).

In the present investigation, the results on somatic embryogenesis have shown that auxin, such as 2,4-D along with cytokinin BA are essential for inducing the somatic embryogenesis from leaf explants of *R. vesicarius.* The auxin/ auxin in combination with cytokinin used in the medium can greatly influence the frequency of induction and also on maturation of somatic embryos. The requirement of cytokinin in addition to auxin was observed in *Solanum surattense* (Rama swamy et al., 2005) whereas, somatic embryogenesis was reported on

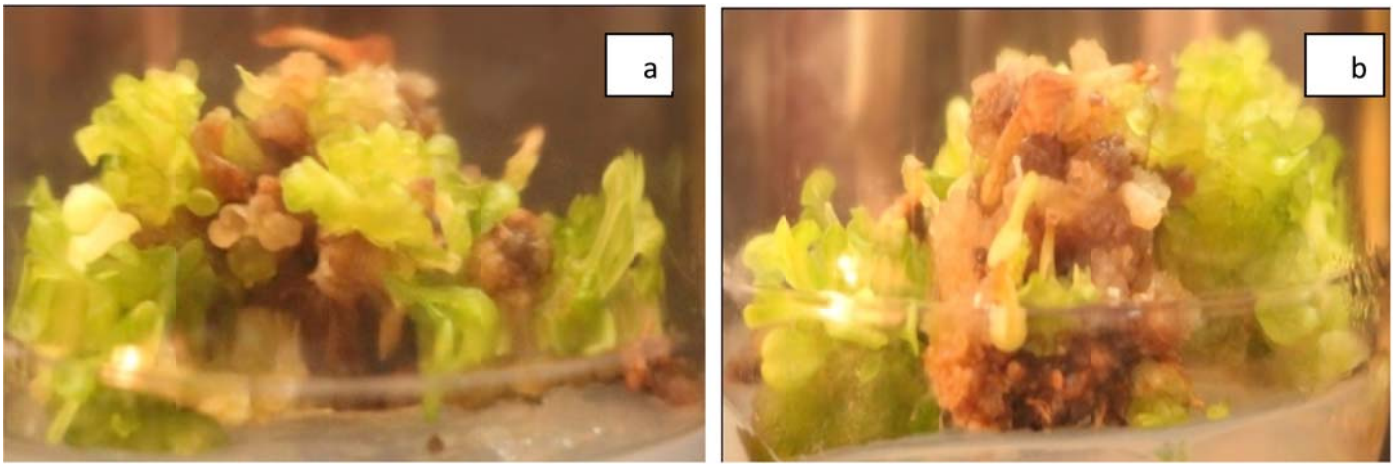

Figure 3. *In vitro* regeneration *via* Somatic embryogenesis in leaf explant cultures of *Rumex vesicarius* L. **(a and b):** Regeneration of shoots from cotyledonary stage embryos in media containing 0.5 mg/l 2,4-D + 2.0 mg/l BA.

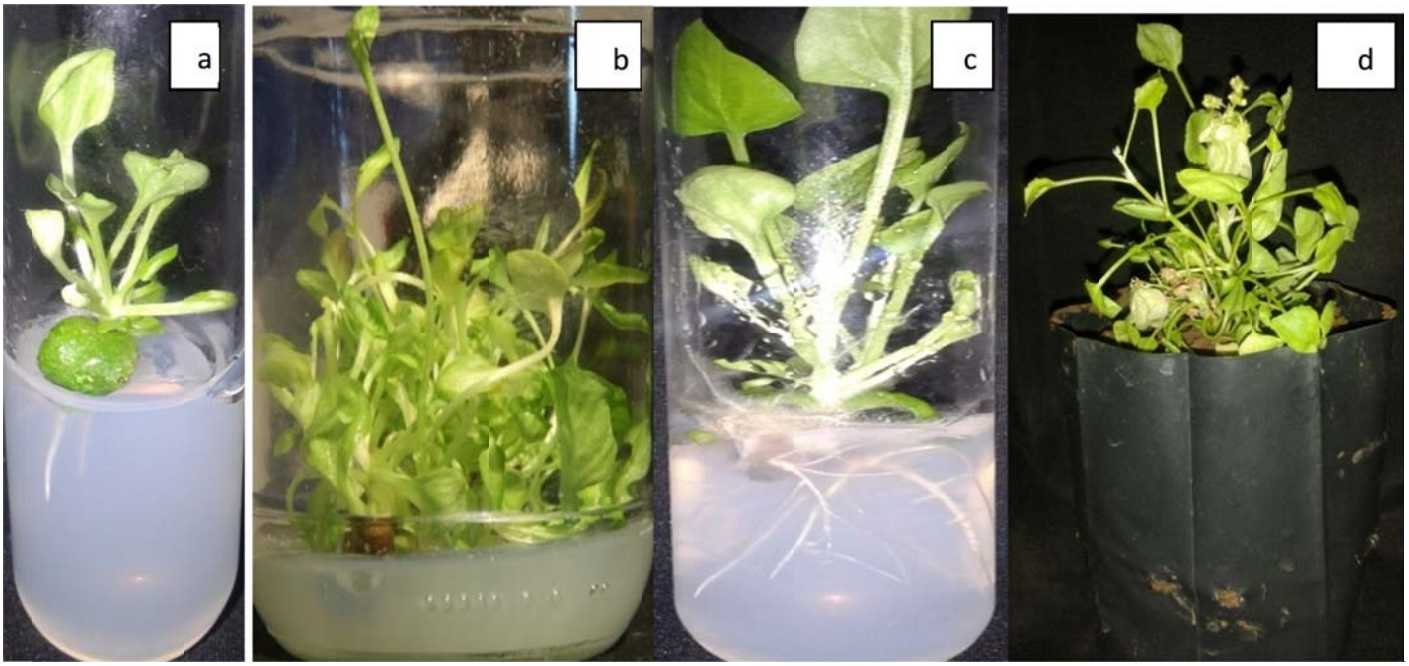

Figure 4. *In vitro* regeneration *via* Somatic embryogenesis in leaf explant cultures of *Rumex vesicarius* L. (**a and b).** Elongation and multiplication of shoots regenerated from somatic embryos in *Rumex vesicarius* L. **(c).** Rooting from *in vitro* regenerated shoots in media containing 1.0 mg/l BA. **(d).** Acclimatization of plantlet.

Table 3. Effect of IBA on induction of roots from shoots in *R. vesicarius* L.

Growth regulators mg/l IBA	Percentage of rooting	Average number of roots from *in vitro* shoots (Mean ± SE)
0.5	60	3.33^{A} ± 0.423
1.0	90	6.38^{C} ± 0.687
2.0	75	4.8^{B}± 0.554

Values are expressed as mean± SE (n=10 in replicate). Mean followed by same letters do not differ significantly at p≥ 0.05 by Tukey's HSD test.

medium containing 2,4-D alone in *Capsicum annuum* L. (Marla et al., 1996). Direct somatic embryogenesis was also reported by adding Kn to the medium and also the number of embryos further increased by enriching the medium with 2,4-D in leaf explants of *Cicer arietinum* L. (Dinesh et al., 1994).

New gene products are needed for the progression from the globular to the heart shaped stage and these new products are synthesized only, when exogenous auxin is removed (Zimmermman 1993). But according to our observation in *R. vesicarius* for morphogenesis of somatic embryos, auxins and cytokinins combination is required. At higher concentration of auxin, probably the population of embryogenic cells drops due to their disruption and elongation and the embryogenic potential of the culture are lost (Aboshama, 2011). Maturation process is critical step in somatic embryogenesis. Similarly, somatic embryo maturation on MS medium containing the combination of 2,4-D and Kn was observed in *Brassica oleraceae* and *Oryza sativa L* (Siong et al., 2011; Verma et al., 2011).

Conclusion

For induction of *in vitro* somatic embryogenesis, the type of primary explants, choice of genotypes and hormonal concentration plays an important role (Josephina and Van Staden, 1990). During the present investigation it was found that the high concentration of auxin in combination with less concentration of cytokinin induced the somatic embryogenesis and maturation of somatic embryos in *R. vesicarius*. However, for germination of somatic embryos, low level of auxin and high concentrations of cytokinin combination is required. Secondary embryogenesis observed in *R. vesicarius* has great potential for its mass propagation and repetitive embryogenesis can also be used for synthetic seed production and genetic transformation.

Conflict of Interest

The author(s) have not declared any conflict of interest.

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