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ORIGINAL PAPER

# Micropropagation protocol for *Salvadora oleoides*

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**Abstract** *Salvadora oleoides* Decne. is a pharmaceutically important plant. Owing to poor seed formation, viability and, germination, and to anthropogenic disturbances, this species is on the verge of extinction. A reproducible micropropagation protocol to increase the population through tissue culture has been standardized and the results are reported here. Callus tissues were initiated from young leaves and stem explants. Leaf calluses proliferated with 1.5 mg/L BAP and 0.9 mg/L 2, 4-D with additives and continuous slow proliferation up to 15 weeks on 0.5 mg/L BAP and additives with 200 mg/L activated charcoal. Direct shoot initiation took place from stem node explants after 12 days; 4–5 shoots per node were produced in 30 days. Shoot clumps elongated and grew further on MS media supplemented with 2 mg/L BAP, 0.2 mg/L NAA and additives, which generated 20–23 shoots. The elongated shoots induced tap roots with 4 mg/L NAA and 200 mg/L activated charcoal in 12 days. In vitro raised plants produced secondary roots when transferred to pots containing vermiculite maintained at 28–35 °C. The plantlets successfully acclimatised in pots containing soil in natural conditions.

**Keywords** *Salvadora oleoides* · Endangered species · Micropropagation · Explants · Calluses · Acclimatize

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## Introduction

*Salvadora oleoides* Decne. belongs to the family Salvadoraceae, and is known as "Jhal" and "Pilu" in Hindi. It occurs in arid, alkaline environments of northwest India (Orwa et al. 2009; Singh 2004). *S. oleoides* is a perennial shrub with a short, twisted trunk bearing globose, eatable, sweet fruit due to the presence of glucose, fructose, and sucrose. The roots are deep and the species is advantageous for shelterbelts and windbreaks in arid and semi-arid regions. It has been reported that camels cherish young branches and leaves as their preferred food because of their relatively high water content (15–36%) (Korejo et al. 2010). The leaves act as a laxative for horses. One of the benefits of this species is that whole plant parts have medicinal properties in various forms (juice, paste, ash) to treat different aliments such as enlarged spleens and fevers, and also possess analgesic properties to control coughs (Arora et al. 2014). The plant parts possess anti-inflammatory, anti-ulcer substances which are also effective in controlling blood glucose levels and improve lipid profiles in euglycemic and anti-diabetic conditions (Yadav et al. 2008). The presence of phytochemical compounds like terpenes, glycosides, steroids, alkaloids tannins and saponin in the stems (Yadav et al. 2005) and roots have highlighted their antimicrobial activities (Kumar et al. 2012)

and an extract from its latex is used in treating sores. The seeds contain 40–45% oil (Zodape and Indusekhar 1997) composed of lauric and myristic acids used in stomach disorders and rheumatic pain (Phulwaria et al. 2014). The oil also possesses larvicidal activities, and provides 100% toxicity to *Anopheles stephensi* larva (a primary mosquito vector of malaria in India). However, the seeds are viable for only 4–5 days and are vulnerable to fungal infection and insect attack, the consequence of which is insufficient germination. Because of this, deforestation by anthropogenic activities and poor natural propagation, its number has declined in the last few years (Kumar et al. 2016). However, *S. oleoides* has the potential to regenerate vegetatively through coppicing, and natural layering by root suckers (Shekhawat et al. 2012). Since the species has been listed as threatened, its daily usage may lead to its extinction shortly. To tackle such as low germination, propagation through tissue cultures seems an excellent method to for the large-scale production of plants in shorter times irrespective of seasonal constraints. Tissue cultures (micropropagation) will permit the rapid multiplication of this species. In this study, we standardized the protocol for callus induction from leaf and stem explants, and the proliferation of leaf calluses. A protocol was developed for plant regeneration from nodal stem explants. As callus tissue is an excellent source of genetic variability, it may be induced to form somatic embryos called synthetic seed. The whole plant may be regenerated and a secondary metabolite extracted without harming the plant.

## Materials and methods

### Explant collection and sterilization

Fresh leaf and stem sprouts were collected from the main campus of the Central University of Punjab, Bathinda. These explants were washed thoroughly with tap water for 5 min, and cuttings with without or with one or two nodes were cut into 1 cm pieces and sterilized. The leaves were first sterilized and then cut into 1 cm discs, washed with 0.5% (v/v) polysorbate 80 (polyoxyethylene sorbitan monooleate, Tween-80) for 3 min and then washed with distilled water until all foam disappeared. The explants were treated with 1.5% (v/v) NaOCl (sodium hypochlorite) for 7 min, and rinsed with distilled water five times under a sterilized laminar air flow chamber. Finally, explants were sterilized with 0.1% (w/v) $HgCl_2$ (mercuric chloride) for 5 min and again rinsed with distilled water five times under sterilized laminar air flow chamber.

### Media preparation, inoculation and incubation conditions

A stock solution of macronutrients, micronutrients, vitamins, and amino acids was prepared for MS media (Murashige and Skoog 1962) and stored at 4 °C. MS media with 3% (w/v) sucrose, pH 5.8 and 0.8% agar for solidification were used. The different concentrations varied from 0.1 to 10 mg/L 2,4-dichlorophenoxy acetic acid (2,4-D), 6-benzyl amino purine (BAP), kinetin (Kn), naphthalene acetic acid (NAA) and/or indole acetic acid (IAA) were used for callus induction, callus proliferation, shoot induction, multiplication, elongation and root induction. Additives (50 mg/L ascorbic acid, 25 mg/L adenylyl sulphate, 25 mg/L arginine and 25 mg/L citric acid) were used in the MS media to determine their effect on micropropagation. The medium was sterilized by autoclaving at fifteen PSI for 15 min. The explant for callus initiation, the 1 cm leaf discs and stem sections without nodes were used for inoculation. Stems with 1 or 2 nodes were inoculated for callus and direct shoot induction. For callus proliferation, the selected calluses were transferred on MS media containing 1 and 1.5 mg/L BAP and 0.7, 0.9 and 1 mg/L NAA and 2, 4-D. For root induction, shoots 2.5–3.5 cm were used. Subculturing was performed after 15 days. All incubations were done at 28 ± 2 °C under 14-hour photoperiod with light intensity 2000 Lux and a 10-hour dark period.

### Acclimatization and analysis

In vitro raised plants were washed thoroughly with running tap water and transferred on tap water-soaked cotton to a covered beaker at 28 ± 2 °C. After 3 days, these were placed in sterilized vermiculite supplemented with 0.1% Hoagland solution without cover under the same temperature and light conditions for 15 days. These were then transferred to pots filled with a mixture of 40% sand, 20% soil, 20% vermiculite and 20% vermicompost to a greenhouse maintained at 28–35 °C. After 10 days, the pots were placed in an open field under natural conditions for acclimatization. On average, twenty replicates were kept per treatment, and each experiment was performed three times and the standard deviation calculated. The expression bellow calculates the percentage response:

$$\text{Percent response} = \frac{(\text{Number of explants showing response})}{(\text{Total number of explants inoculated})} \times 100$$

## Results

### Callus induction and proliferation

MS media fortified with additives, 2.5 mg/L BAP and 1 mg/L NAA, resulted in 85% callus induction from leaves (Fig. 1a) after 13 days. While 2.5 mg/L 2, 4-D and 2 mg/L BAP induced 100% callus after 10 days but increased concentration of 2, 4-D up to 3 mg/L and 2 mg/L BAP induced 100% callus (Fig. 1b) after 8 days. However, in Fig. 1c, we kept the stems horizontally on media with a concentration of 2.5 mg/L BAP, 0.2 mg/L NAA in addition to additives, while stems placed vertically on the same media (Fig. 1d) showed equal and excellent responses after 23 days. The percent response with other concentrations of BAP, 2, 4-D, NAA, and IAA are shown in Table 1. The proliferation of callus is mentioned in Table 2, in which 1.5 mg/L BAP, 0.7 mg/L NAA or 2, 4-D and additives

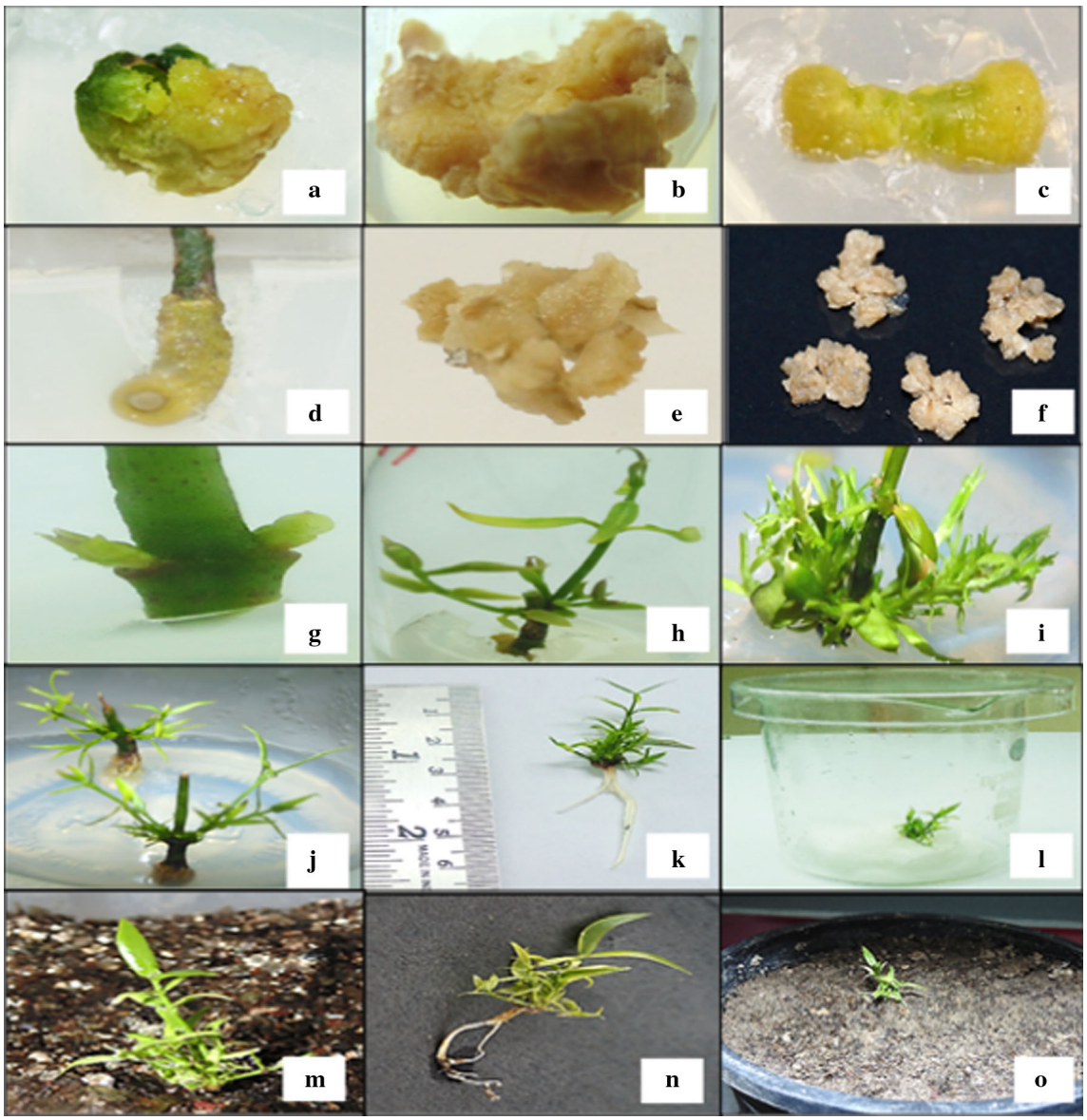


**Fig. 1** Callus formation from young sprouts and regeneration from stem young nodes: **a–d** callus induction: **a** from leaf on MS + 2.5 mg/L BAP + 1 mg/L NAA + additives, **b** from leaf on MS + 2 mg/L BAP + 3 mg/L 2, 4-D + additives; **c** from horizontal inoculated stem and **d** from vertical inoculated stem, on 2 mg/L BAP + 3 mg/L 2, 4-D + additives. **e** Leaf callus proliferation on MS + 1 mg/L BAP + 0.9 mg/L 2, 4-D + additives. **f** Leaf callus proliferation on MS + 0.5 mg/L BAP + additives + 200 mg/L Activated Charcoal. **g** Shoot induction, **h** shoot multiplication, **i** further shoot multiplication of shoot on 2 mg/L BAP + 0.2 mg/L NAA + additives. **j** Shoot elongation on 2.5 mg/L BAP + 0.2 mg/L NAA + additives. **k** Root induction on MS + 4 mg/L NAA + 200 mg/L Activated Charcoal. **l–o** Acclimatization: **l** plant in water soaked cotton, **m** Plantlet in vermiculite, **n** induction of secondary roots and, **o** Plant in natural condition (pot)

**Table 1** Effect of different plant growth regulators (PGR) on callus induction from young leaf and stem explants

| Explant | PGR (mg/L) | | | | | Additives | Number of days | Callus induction response (Mean ± SD) | Percent response |
|---|---|---|---|---|---|---|---|---|---|
| | BAP | 2, 4-D | IBA | NAA | IAA | | | | |
| Leaf | 2 | 3 | – | – | – | + | 8 | 20 ± 0 | 100 |
| | 2 | 2.5 | – | – | – | + | 10 | 20 ± 0 | 100 |
| | 2.5 | – | 0.5 | – | 0.5 | + | 15 | 5 ± 1 | 25 |
| | 2.5 | – | 1 | 1 | – | + | 23 | 16 ± 1 | 80 |
| | 5 | – | 2 | 2 | – | + | 12 | 16 ± 0 | 80 |
| | 2 | – | – | 1 | – | + | 20 | 2 ± 1 | 10 |
| | 2.5 | – | – | 1 | – | + | 13 | 17 ± 0 | 85 |
| Stem | 2 | – | – | 2 | – | – | 30 | 5.33 ± 0.57 | 26.67 |
| | 2.5 | – | – | 0.5 | 0.1 | – | 35 | 4 ± 0 | 20 |
| | 2 | – | – | 0.2 | – | + | 30 | 2.7 ± 0.58 | 13 |
| | 2.5 | – | – | 0.2 | – | + | 23 | 9.3 ± 0.58 | 46.67 |

**Table 2** Effect of different plant growth regulators (PRG) on proliferation of induced callus from leaves

| PGR (mg/L) | | | Additives | Days to proliferation |
|---|---|---|---|---|
| BAP | NAA | 2, 4-D | | |
| 1 | 0.7 | – | + | 10 |
| 1.5 | – | 0.7 | + | 10 |
| 1.5 | – | 0.9 | + | 6 |
| 1.5 | – | 1 | + | 6 |

started callus proliferation after 10 days of inoculation. Increase in concentration of 2, 4-D up to 1 mg/L initiated proliferation after 6 days (Fig. 1e). The proliferated callus sub-cultured on MS media with 0.5 mg/L BAP, additives and 200 mg/L activated charcoal showed low proliferation up to 15 weeks (Fig. 1f).

### Shoot induction, multiplication, and elongation

Direct shoot induction recorded as bud initiation from stem nodes on MS media containing additives are given in Table 3. The highest response of 86.7% was with 2.5 mg/L BAP and 0.2 mg/L NAA after 12 days (Fig. 1g). For multiplication, 2.5 mg/L BAP with 0.2 mg/L NAA provided the best response of 4–5 shoots per node with 76.7% multiplication in 30 days (Fig. 1h and Table 4). Further subculturing of a multiplied shoot clump of set 2 on 2 mg/L BAP with 0.2 mg/L NAA for 30 days induced 36.7% response with 20–23 shoots in clumps (Fig. 1i and Table 4). However, the percent response was less as compared to 76.7% but subculturing and change of in media concentration might have prompted vigorous multiplication of shoots which led to enhance in shoot multiplication. Shoot elongation on 2.5 mg/L BAP and 0.2 mg/L NAA produced a maximum response of 83.3% with shoots reaching 2.5–3.5 cm in length in 30 days (Fig. 1j and Table 5).

### Root induction and acclimatization

MS media with 4 mg/L NAA induced tap roots from elongated shoots (Fig. 1k) provided 16.7% response after 12 days with mean ± SD value (3.33 ± 0.58). Initially, in vitro plantlets survived on water soaked cotton swabs inside a beaker (Fig. 1l), and secondary root formation was induced after shifting to vermiculite during hardening (Fig. 1m, n). Acclimatization was successful when plants were shifted from vermiculite to potted soil (Fig. 1o).

## Discussion

*S. oleoides* Decne. is a small bushy tree growing in arid and alkaline conditions in northwest India. This endangered species has several medicinal and pharmaceutical properties (Singh 2004; Arora et al. 2014; Yadav et al. 2008), making it economically important and requiring a targeted effort for its quick multiplication. The present investigation was intended to develop a reproducible protocol for the fast multiplication of this species through tissue culture.

Successful multiplication of woody plants through tissue culture depends on several important factors including the type of explants used, seasonal factors, the age of explant (Shekhawat et al. 1993), the media used for callusing, shoot initiation, and rooting, as well as a suitable mix of plant growth regulators and additives.

**Table 3** Effect of plant growth regulators on bud formation from young node stem explants

| BAP (mg/L) | Kn (mg/L) | NAA (mg/L) | Additives | Number of days | Shoot bud induction response (mean ± SD) | Percent response |
|---|---|---|---|---|---|---|
| 1 | – | – | + | 22 | 8 ± 0 | 40 |
| 2 | – | – | + | 20 | 6 ± 0 | 30 |
| – | 2 | – | + | 35 | 2 ± 0 | 10 |
| – | 4 | – | + | 32 | 2 ± 1 | 10 |
| 1 | – | 0.2 | + | 18 | 5.33 ± 0.58 | 26.67 |
| 2 | – | 0.2 | + | 14 | 8 ± 1 | 40 |
| – | 2 | 0.2 | + | 30 | 2 ± 1 | 10 |
| – | 2.5 | 0.2 | + | 30 | 2 ± 1 | 10 |
| 2.5 | – | 0.2 | + | 12 | 17.33 ± 0.58 | 86.67 |

**Table 4** Effect of BAP and NAA on multiplication of induced shoots

| Number of days | | BAP (mg/L) | NAA (mg/L) | Additives | Shoot bud multiplication (mean ± SD) | Percent multiplication | Number of shoots |
|---|---|---|---|---|---|---|---|
| 30 days | Set-1 | 2 | 0.2 | + | 14 ± 0 | 70 | 4–5 |
| | Set-2 | 2.5 | 0.2 | + | 15.33 ± 0.58 | 76.67 | 4–5 |
| Sub cultured for next 30 days from set-2 | | 2 | 0.2 | + | 7.33 ± 0.58 | 36.67 | 20–23 |

**Table 5** Effect of plant growth regulators on elongation of multiplied shoots

| BAP (mg/L) | IAA (mg/L) | NAA (mg/L) | Additive | Multiple shoot elongation (mean ± SD) | Percent response | Shoot length (cm) in 30 days |
|---|---|---|---|---|---|---|
| 1 | – | – | + | 15 ± 1 | 75 | 2–2.5 |
| 1 | 0.5 | – | + | 15 ± 0 | 80 | 2 |
| 1.2 | 0.2 | – | + | 14 ± 1 | 70 | 2 |
| 2 | – | – | + | 16.67 ± 0.58 | 83.33 | 2–3 |
| 1 | – | 0.2 | + | 10.67 ± 0.58 | 53.33 | 2–3 |
| 2 | – | 0.2 | + | 13.33 ± 0.58 | 66.67 | 2–2.5 |
| 2.5 | – | 0.2 | + | 16.67 ± 0.58 | 83.33 | 2.5–3.5 |

No previous studies have reported induced callus from leaves of this species. Our main objective of this study was to induce callus, for which we used different concentrations of growth regulators and additives. It has been observed that the auxin 2, 4-D is responsible for the induction of soft callus tissue (Daffalla et al. 2011). In our case, MS media with cytokinin (BAP or Kn) and auxin (2, 4-D) induced callus. However, BAP was more effective than Kn with auxin (IAA, NAA and 2, 4-D) in callus formation in cotyledons of *Salvadora persica* (Kumar et al. 2012). Leaves as explants produced callus that was non-organogenic which, on further subculturing, becomes brown and declines to grow (Mathur et al. 2002). However in our study, due to regular subculturing and reduction of hormonal concentrations, callus was maintained and proliferation observed. This might be that subculturing removes bioactive compounds (Gonzalez et al. 2011) such as phenolic substances (Hoque and Arima 2002). Bioactive compound get accumulated in in vitro callus culture like flavonoid, stilbenes, sterols and phenolic acid with time period (Castro et al. 2016).

The placement and exposure of explants in the media also affects the success rate. In this study, inoculation of stems vertically and horizontally on the media resulted in a healthy amount of callus. Various reports are available on *Balanites aegyptiaca* (Ndoye et al. 2003), *Sapindus trifaliatus* (Asthana et al. 2011) and *S. persica* (Kumar et al. 2012) regarding callus formation at the basal ends of

explants. This may be due to the accumulation of auxin. In our study, callus was observed but did not proliferate long in respective media and dried. However, callus formation (100%) from leaves using MS media with BAP, 2, 4-D and additives, and callus proliferation on MS media containing additives with BAP (1.5 mg/L) and 2, 4-D (0.9 mg/L) were recorded in this study.

The young explants and vertical placement of stems of *S. persica* were more useful for micropropagation (Phulwaria et al. 2011). In another study with *Tecomella undulata* (Sm.) Seem. vertical placement of nodal stems for culturing of was more useful in bud breaking and shoot production because this positioning avoided callus formation as it kept the nodal portion away from direct contact with the medium (Rathore et al. 1991). BAP has the ability to initiate shoot induction over Kn from node stems used as explants in *S. persica* (Phulwaria et al. 2011), and *S. oleoides* (Kumar et al. 2016). Plant tissues metabolize BAP more easily than other synthetic PGRs (Rai et al. 2010). Combinations of cytokinin and NAA produced maximum shoots in our study and are in agreement (Phulwaria et al. 2011) but reduced shoot induction due to callus formation and proliferation at the base of shoot clumps in their studies. Similarly, the addition of NAA induced shoot multiplication of *S. persic* (Phulwaria et al. 2014) and *S. oleoides* (Kumar et al. 2016), but in our case BAP with NAA induced multiplication of shoots and also induced callus tissue at the base of the explants. The shoot bud initiation (86.7%) from stem nodes, multiplication (76.7%) and elongation (2.5–3.5 cm) on media having mixed concentration of BAP, NAA and additives were observed in our study.

We observed that tap root formation occurred with NAA (4 mg/L). It might be that auxin (NAA) has a stimulatory effect on root formation and elongation. Reports suggest that a high number of roots is important for the establishment of tissue cultured plantlets (Sharma et al. 2007). According to reports, 80% root induction was obtained with a 1 mg/L NAA treatment (Kumar et al. 2016). The same results were observed in our study as tap roots were induced after NAA treatment but the percentage was low. Secondary roots were induced when the plantlets with tap roots were shifted to pots containing vermiculite and kept under controlled conditions in a growth house.

Further work will be required to commercialize the protocol and to make it more practical and reproducible under natural conditions. The present communication has been designed with an aim to develop high-frequency callus regeneration from leaves and stems.

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