In vitro culture, plant regeneration and clonal behaviour of *Sesuvium portulacastrum* (L.) L. : a prospective halophyte

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ABSTRACT

An efficient plant regeneration protocol using axillary shoots of the salt accumulator halophyte, *Sesuvium portulacastrum* (L.) L. was established and *in vitro* responses of six *Sesuvium* clones were studied. The shoot and root induction responses to cytokinins and auxins in clone MH (Maharashtra) were concentration specific. Significantly the highest number of shoots, average shoot elongation and percent shoot regeneration per explant were observed on MS medium supplemented with 40 μ M 2-isopentenyl adenine (2iP) followed by 20 μ M benzyladenine (BA). Higher cytokinin (60 μ M), however, inhibited shoot induction and shoot length. The lower concentrations (5 or 10 μ M) of α -naphthaleneacetic acid (NAA) proved more effective for root induction, number of roots and average root length. Well-developed plantlets were successfully hardened and established in the field with more than 85 % survival rate. *In vitro* response of six *Sesuvium* clones cultured on MS + 20 μ M BA revealed higher multiplication rate in clone MH and KA (Karnataka, India) compared to other clones. The results offer the prospect of selecting clones of this species with characteristics desirable for utilization and/or restoration in specific ecological zones. [Physiol. Mol. Biol. Plants 2010; 16(2) : 187-193] E-mail : prasanna@barc.gov.in

Keywords : *Sesuvium portulacastrum*, tissue culture, regeneration, restoration, clonal responses

INTRODUCTION

Sesuvium portulacastrum (L.) L. commonly called seapurslane, is an important perennial psammophytic halophyte of Aizoaceae. It grows in the subtropical, mediterranean, coastal and warmer areas around the world (Balasubramanian *et al.*, 2006). In India, it grows in the coastal sides of eastern and western regions (Lokhande *et al.*, 2009) in association with the true mangroves. Of the various categories of mangroves, *Sesuvium* comes under “salt accumulators”. These species accumulate high concentration of salt in cells and tissues and overcome salt toxicity by developing succulence (Lüttge *et al.*, 1988). Environmentally, the plant has a remarkable ability to survive under different abiotic stress including salinity, heavy metals and drought (Slama *et al.*, 2008).

Effect of anthropogenic factors on the growth, morphology and genetic makeup of *Sesuvium* clones growing in a specific area have been reported (Lokhande *et al.*, 2009). Such genetic as well as morphological variations do affect the functioning of the marsh ecosystem (Selisker *et al.*, 2002; Wang *et al.*, 2003). Apart from survival in the saline habitat, proliferation of *Sesuvium* is hindered because of insufficient planting material and unfavourable environmental conditions like high salinity, high wind velocity, temperature variation and muddy anaerobic soil (Kathiresan *et al.*, 1997). *In vitro* culture techniques offer tools for germplasm conservation and genetic improvement (Xu *et al.*, 2008) besides producing a large number of plantlets for cultivation in saline regions. The system would also facilitate physiological studies on mechanism of salt tolerance as reported in halophytes such as *Spartina patens* (Wu *et al.*, 1998), *Suaeda nudiflora* (Cherian and Reddy, 2003), *Crithmum maritimum* (Amor *et al.*, 2005) and *Thellungiella halophila* (Zhao *et al.*, 2009). The present investigation was aimed at developing an

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efficient protocol for micropropagation of *Sesuvium* by using axillary buds and studying the response of different *Sesuvium* clones to shoot regeneration.

MATERIALS AND METHODS

Collection, establishment and authentication of plant material

Six clones (MH, TN, KA, GJ, AP and OR) of *Sesuvium portulacastrum* (L.) L., collected from geographically distant coastal regions in India (Table 1) were planted in soil beds (4' × 2') and maintained in the Botanic garden at the Department of Botany, University of Pune (lat. 18° 31' N long. 73° 51'), India (Lokhande *et al.*, 2009). The identification and authentication of plant material was by Botanical Survey of India (BSI) and the specimen samples are deposited in the BSI (Ref.: BSI/WC/Tech/2007/904).

Source of explant, sterilization and culture conditions

The nodal explants (~ 10 mm, from field grown plants) were washed with tap water and disinfected using 1 % (w/v) Sovistin solution with the addition of two drops of Tween-20 for 30 min. The explants were rinsed thrice with sterile distilled water and surface sterilized with 0.1 % (w/v) mercuric chloride (HgCl₂) for 5 min followed by washing with sterile distilled water 5 times. The explants were placed vertically onto the MS (Murashige and Skoog, 1962) medium containing 3 % sucrose and 10 µM BA. The pH of the medium was adjusted to 5.8 and sterilized by autoclaving at 121 °C for 15 min. The medium was solidified with 0.8 % agar-

agar (Himedia Pvt. Ltd., India). The cultures were maintained at 25 ± 2 °C and 16 h photoperiod under cool white fluorescent light (Philips India, Ltd., 40 µmol m⁻² s⁻¹) and 70 % relative humidity. After one month, axillary shoots induced from nodal explants were excised and maintained on freshly prepared shoot induction medium (MS + 10 µM BA). The axillary shoots were regularly sub-cultured at one month interval for one year. Among the six clones, the axillary shoot cultures of clone MH were used to study the interactive effect of cytokinins and auxins on organogenesis.

Shoot organogenesis

The single axillary shoots of clone MH were excised and cultured individually on MS solid medium containing 3.0 % sucrose supplemented with BA, Kin, TDZ or 2iP (0, 10, 20, 40 or 60 µM). Data were recorded on number of shoots per explant, percent explants forming shoots, average shoot length (mm), number of roots per explants, percent shoots forming roots and average root length (mm) from 20 explants replicated at least thrice.

In vitro rooting

Vigorously growing axillary shoots of clone MH induced on MS + 40 µM 2iP or MS + 20 µM BA were subjected to root induction in MS liquid medium supplemented with NAA or IAA (0, 2.5, 5.0, 10 or 20 µM). After one month, rooted plantlets were sub-cultured to ½ strength MS liquid medium (only minerals) for 15 days. These plantlets were further acclimatized under *ex vitro* conditions.

Table 1. Description of different *Sesuvium* clones collected from coastal regions of India.

Sr. No.	Date of Collection	State	Code	Collection site (City/village)	Latitude °N	Longitude °E	Properties of the clones*		
							Stem colour	Flower colour	Leaf size
1	10/02/07	Maharashtra	MH	New Mumbai	19° 02'	73° 01'	Green	Pink	Small
2	24/04/07	Tamilnadu	TN	Kanyakumari	8° 10'	77° 26'	Green	Pink	Large
3	29/04/07	Karnataka	KA	Mangalore	12° 52'	74° 49'	Pink	Pink	Medium
4	03/06/07	Gujarat	GJ	Jamnagar	22° 28'	70° 04'	Red	White	Small
5	14/06/07	Andhra Pradesh	AP	Kakinada	16° 57'	82° 15'	Pink	Pink	Medium
6	17/06/07	Orissa	OR	Dhamra	20° 48'	86° 56'	Red	Pink	Small

*For the properties of clones see Lokhande *et al.*, 2009

Acclimatization of rooted plantlets to *ex vitro* conditions

The *in vitro* grown, well developed rooted plantlets were transferred to plastic pots (5 × 7 cm) containing sterilized sand:soil (1:1) and allowed to grow for 15 days in the culture room. The pots were covered with transparent polythene bags to maintain the relative humidity. Subsequently, the pots were transferred to greenhouse under natural day/night photoperiod, 25 - 30 °C and 60-70 % relative humidity. After three months, the plantlets were approximately 100 mm tall and ready for further transplantation to bigger plastic pots (30 × 20 cm). The plants were then transferred to field conditions.

Clonal micropropagation

The axillary shoots induced on multiple axillary shoot induction medium (MS + 10 µM BA) and maintained for one year by regular sub-culturing on the same medium at the interval of one month each were used as a source of explants to study the response of all the six *Sesuvium* clones to micropropagation. The axillary shoots of each clone were cultured separately on optimized shoot multiplication medium (MS + 20 µM BA).

Statistical analysis

The experiments were laid out in completely randomized design (CRD), with 20 explants per treatment. All the

Table 2. Effect of various concentrations of cytokinins (BA, Kin, TDZ or 2iP) on shoot multiplication in axillary shoot explants of *Sesuvium portulacastrum* clone-MH.

Concentrations of cytokinins in media (µM)				Number of shoots per explant	Percentage of node forming shoots (%)	Average shoot length (mm)	Number of roots per explant	Percentage of shoots forming roots (%)	Average root length (mm)
BA	Kin	TDZ	2iP						
0.0	0.0	0.0	0.0	1.40 ± 0.11 ^{j,k}	40 ^{c,d}	14.65 ± 0.10 ^m	12.93 ± 0.20 ^a	80 ^a	38.75 ± 0.21 ^a
10	0.0	0.0	0.0	3.70 ± 0.15 ^b	100 ^a	29.30 ± 0.21 ^c	NR	NR	NR
20	0.0	0.0	0.0	4.95 ± 0.21 ^{ECa}	100 ^a	27.50 ± 0.19 ^d	NR	NR	NR
40	0.0	0.0	0.0	2.66 ± 0.10 ^{e,f}	75 ^{a,b}	24.65 ± 0.10 ^f	NR	NR	NR
60	0.0	0.0	0.0	2.00 ± 0.00 ^h	65 ^{b,c}	18.70 ± 0.21 ^h	NR	NR	NR
0.0	10	0.0	0.0	1.50 ± 0.10 ^{i,j}	35 ^{d,e}	17.42 ± 0.20 ^j	2.70 ± 0.10 ^d	50 ^b	21.20 ± 0.21 ^c
0.0	20	0.0	0.0	2.42 ± 0.11 ^{f,g}	35 ^{d,e}	15.57 ± 0.11 ^l	3.45 ± 0.20 ^c	80 ^a	24.80 ± 0.16 ^b
0.0	40	0.0	0.0	2.90 ± 0.07 ^{d,e}	40 ^{c,d}	15.37 ± 0.10 ^l	5.17 ± 0.16 ^b	30 ^c	14.33 ± 0.10 ^d
0.0	60	0.0	0.0	3.30 ± 0.10 ^c	30 ^{d,e}	14.50 ± 0.08 ^{m,n}	1.95 ± 0.07 ^e	10 ^e	11.00 ± 0.00 ^e
0.0	0.0	10	0.0	1.10 ± 0.06 C ^k	10 ^e	16.20 ± 0.32 ^k	NR	NR	NR
0.0	0.0	20	0.0	1.30 ± 0.10 C ^{j,k}	30 ^{d,e}	17.90 ± 0.33 ⁱ	NR	NR	NR
0.0	0.0	40	0.0	1.80 ± 0.13 C ^{h,i}	70 ^b	21.25 ± 0.35 ^g	NR	NR	NR
0.0	0.0	60	0.0	1.20 ± 0.08 C ^{j,k}	20 ^{d,e}	14.30 ± 0.21 ⁿ	NR	NR	NR
0.0	0.0	0.0	10	2.10 ± 0.71 C ^{g,h}	75 ^{a,b}	26.85 ± 0.43 ^e	NR	NR	NR
0.0	0.0	0.0	20	3.70 ± 0.10 C ^b	100 ^a	36.30 ± 0.69 ^b	NR	NR	NR
0.0	0.0	0.0	40	5.20 ± 0.15 C ^a	100 ^a	47.95 ± 0.52 ^a	NR	NR	NR
0.0	0.0	0.0	60	3.10 ± 0.22 C ^{c,d}	85 ^{a,b}	14.30 ± 0.21 ⁿ	NR	NR	NR

Data are mean ± SE scored after 30 days from 20 axillary shoot explants per treatment and repeated thrice. Mean followed by same letter are not significantly different at $P \leq 0.001$ level by Duncan's multiple range test. NR: No Response for rooting; C: Callus induction at cut end of shoot; EC: Extensive callus induction.

experiments were repeated thrice. The data were analyzed by one-way analysis of variance (ANOVA) using statistical software, General Linear Model procedure (SPSS 10.0 statistical package). The treatment means were compared by using the post hoc least significant difference (LSD) test. Means differing significantly were compared using Duncan's Multiple Range Test (DMRT) at $P \leq 0.001$ probability level. Data variability was expressed as the mean $\pm$ SE.

RESULTS

Influence of cytokinins on shoot organogenesis

The induction of high frequency of axillary shoot formation from the nodal explants was observed in all the *Sesuvium* clones on MS + 10 μ M BA. These axillary shoots appeared to proliferate directly from node via axillary branching of buds from the explants.

The single axillary shoot of clone MH grown on MS supplemented with different cytokinins (BA, Kin, TDZ or 2iP) resulted in an increased number of axillary shoots per explant (Table 2). Higher concentrations of 2iP (40

μ M) promoted multiple shoot production (Fig. 1A) compared to BA, Kin or TDZ (Table 2), whereas 60 μ M of 2iP significantly inhibited growth and shoot formation frequency (Table 2). BA or 2iP also produced extensive, compact, chlorophyllous callus at the cut ends of the axillary shoot explants.

Effect of auxins on *in vitro* rooting

The axillary shoots cultured on MS basal liquid medium supplemented with NAA or IAA (0, 2.5, 5.0, 10 or 20 μ M) exhibited differential root induction response (Table 3 and Fig. 1B). Addition of NAA (5.0 or 10 μ M) significantly increased root induction frequency, however, the highest number of roots per axillary shoot, root elongation and root induction frequency were observed on the MS medium supplemented with 10 μ M NAA (Table 3 and Fig. 1B). The low (2.5 μ M) and higher (20 μ M) concentrations of NAA significantly reduced the rooting response. Root induction ranged between 70 and 95 % and average number of roots on all the concentrations of NAA (Table 3) was found to be the highest and significantly different as compared to responses on MS basal liquid medium (Table 3) and medium with IAA (Table 3).

Acclimatization of rooted plantlets to *ex vitro* conditions

The plantlets rooted on media containing either 5.0 or 10.0 μ M NAA did not reveal any differences in

Table 3. Effect of different concentrations of auxins (NAA or IAA) on rooting of shoots derived from the axillary shoot explants of *Sesuvium portulacastrum* clone-MH.

Concentrations of auxins in media (μ M)		Number of roots per explant	Percentage of shoots forming roots (%)	Average root length (mm)
NAA	IAA			
0.0	0.0	12.93 $\pm$ 0.21 ^f	65 ^{b,c}	38.75 $\pm$ 0.22 ^a
2.5	0.0	25.94 $\pm$ 0.20 ^c	70 ^{b,c}	25.05 $\pm$ 0.17 ^c
5.0	0.0	29.63 $\pm$ 0.46 ^b	85 ^{a,b}	31.15 $\pm$ 0.22 ^c
10	0.0	37.21 $\pm$ 0.19 ^a	95 ^a	34.89 $\pm$ 0.77 ^b
20	0.0	24.66 $\pm$ 0.36 ^d	75 ^{a,b}	28.60 $\pm$ 0.58 ^d
0.0	2.5	11.13 $\pm$ 0.21 ^h	75 ^{a,b}	18.80 $\pm$ 0.21 ^g
0.0	5.0	20.76 $\pm$ 0.18 ^e	85 ^{a,b}	22.64 $\pm$ 0.20 ^f
0.0	10	12.44 $\pm$ 0.43 ^g	50 ^c	17.33 $\pm$ 0.27 ^h
0.0	20	7.60 $\pm$ 0.37 ⁱ	50 ^c	12.90 $\pm$ 0.37 ⁱ

Data are mean $\pm$ SE scored after 30 days from 20 axillary shoot explants per treatment and repeated thrice. Mean followed by the same letters are not significantly different at $P \leq 0.001$ level by Duncan's multiple range test.

Table 4. Differential responses of axillary shoot explants of different *Sesuvium* clones to BA (20 μ M) induced shoot multiplication.

Clone	Shoots per explant	Explant forming shoots (%)	Shoot length (mm)	Callus induction
MH	4.95 $\pm$ 0.21 ^a	100 ^a	27.50 $\pm$ 0.19 ^a	++
GJ	2.43 $\pm$ 0.41 ^e	65 ^c	23.40 $\pm$ 0.49 ^c	+
KA	4.17 $\pm$ 0.35 ^b	90 ^b	26.20 $\pm$ 0.54 ^b	+++
TN	3.83 $\pm$ 0.35 ^{b,c}	70 ^c	19.17 $\pm$ 0.51 ^c	++
AP	3.53 $\pm$ 0.35 ^{c,d}	85 ^b	23.03 $\pm$ 0.50 ^c	+
OR	3.07 $\pm$ 0.45 ^d	75 ^c	21.23 $\pm$ 0.50 ^d	+

Degree of callus induction: + low; ++ moderate; +++ high; Data are mean $\pm$ SE scored after 30 days from 20 axillary shoot explants per treatment and repeated for thrice. Mean followed by same letters are not significantly different at $P \leq 0.001$ level by Duncan's multiple range test.

acclimatization. A total of 150 plantlets transferred to plastic pots (Fig. 1D) adapted well to *ex vitro* conditions and grew vigorously after transfer to bigger plastic pots (Fig. 1E). After one month, hardened plants were transferred to field conditions; survival rate was greater than 85 % (*i.e.* more than 127 plantlets). The young plants grew vigorously in the field. After three months of acclimatization, all the plants showed similar morphology as their parent plant growing under natural conditions.

Fig. 1. Tissue culture and plant regeneration in *Sesuvium portulacastrum* clone-MH. **(A)** Shoot multiplication from axillary shoot explant on 20 μM BA. **(B)** *In vitro* root induction from axillary shoots on MS liquid medium supplemented with 10 μM NAA. **(C)** Multiple axillary shoots maintained on parental media (20 μM BA) after one month produced 15-25 shoots per culture. **(D)** *In vitro* grown rooted plantlets transferred to small plastic pots for acclimatization. **(E)** Acclimatized rooted plantlets transferred to big plastic pots for further growth in *ex vitro* conditions.

Differential clonal behavior to *in vitro* propagation

The axillary shoot explants of clone MH produced significantly the highest number of multiple shoots on MS + 20 μM BA with 100 % shoot regeneration frequency, whereas the least response was observed in clone GJ- Gujarat (Table 4). Among the clones, multiple shoots of clone MH and KA (Karnataka) grew vigorously. The shoot multiplication rate of clone MH ranged between 15 and 25 shoots per culture after four weeks of serial subculture (Fig. 1C). Though all the clones produced chlorophyllous, compact callus from the cut end of axillary shoot explants, the degree of callus proliferation was clone specific; extensive callus proliferation was observed in the clone KA followed by the clone MH and TN (Table 4), whereas the clone GJ, AP (Andhra Pradesh) and OR (Orissa) showed less callus proliferation.

DISCUSSION

Though vegetative propagation via rooting of cuttings is practiced in *Sesuvium portulacastrum*, development of an efficient protocol for micropropagation is essential for its long term germplasm conservation as well as year round source of plant material on a large scale for restoration of wetlands, desalination of saline soil and phytoremediation of heavy metal contaminated soil in the arid and semiarid regions of the world. In the present investigation, interactive effects of cytokinins (BA, Kinetin, TDZ or 2iP) and auxins (NAA or IAA) were studied on multiple shoot induction and rooting from axillary shoot explants of *Sesuvium*. The responses to cytokinins for shoot growth and multiplication rate were concentration specific.

Among the cytokinins, 2iP exhibited better response, as the number of shoots and average shoot length were the highest with 40 μM 2iP. The higher shoot multiplication efficiency of 2iP was also reported in *Decalepis hamiltoni* (Giridhar *et al.*, 2005) and *Gentiana punctata* (Keul *et al.*, 2005). Thus, our emphasis on number and shoot elongation from axillary shoot along with shoot regeneration frequency are of significance as compared to earlier reports of Kathiresan *et al.* (1997) wherein the highest shoot multiplication rate was observed on MS medium supplemented with 2.0 mg l⁻¹ BA + 0.5 mg l⁻¹ 2,4-D, with little effect on shoot elongation. A good multiplication rate along with proper shoot length is the most important factor in rendering a propagation protocol cost-effective.

Supplementation of BA (20 μ M) to the medium produced maximum number of shoots but the average shoot length was lower than that observed on 2iP, whereas higher concentrations of BA (40 and 60 μ M) progressively reduced the number of shoots and height. Similar results were also observed by Grigoriadou and Maloupa (2008) in the halophyte *Crithmum maritimum*, wherein the lower concentrations of BA resulted in effective microshoot production, and higher concentrations affected rate of microshoot production. Besides, the higher concentration not only inhibited shoot induction but also led to stunted growth in *Malus zumi* shoots (Xu *et al.*, 2008). The low levels of BA also resulted in enhancement of shoot formation frequency of H-grey mangrove- *Avicennia marina* (Abdulaziz and Jammel, 2003). Similar to our results on the effectiveness of 2iP, Joshi and Dhawan (2007) also reported the superiority and the highest shoot bud induction ability of 2iP supplemented medium followed by BA in *Swertia chirayita*. In comparison, Kin showed lower shoot induction and shoot length response. Kinetin didn't promote shoot regeneration frequency in *Cichorium intybus* (Yucesan *et al.*, 2007), *Holarrhena antidysentrica* (Kumar *et al.*, 2005) and *Gymnema sylvestris* (Reddy *et al.*, 1998). TDZ, a potent cytokinin, showed improved shoot multiplication efficiently in most of the plant species (Murthy *et al.*, 1998) however, no such promotive effect was observed in *Sesuvium portulacastrum*. Similar results on induction of adventitious buds in *Fragaria* were also reported by Landi and Mezzetti (2006).

The success of micropropagation relies on rooting percentage and hence survival of plants in the field conditions. Auxin type, concentration and use of a liquid system had a significant effect on rooting of shoots, specifically for early root induction (within 5 days) and elongation. Makunga *et al.* (2006) in *Thapsia garganica* and Jayanand *et al.* (2003) in *Cicer arietinum* found use of liquid culture most effective for rooting. Higher rooting response and subsequent hardening were observed on a MS liquid medium supplemented with 5.0 to 10 μ M NAA, compared to lower (2.5 μ M) or higher (20 μ M) concentrations. Use of IAA induced less rhizogenetic response with poor root regeneration frequency and shorter root elongation. The superiority of NAA over other auxins for rooting has also been previously reported in *Swertia chirayita* (Joshi and Dhawan, 2007), *Juncus roemerianus* and *J. gerardi* (Wang *et al.*, 2005).

Tissue culture responses in several crop plants are known to be genotype dependant. In *Sesuvium*, multiple

shoot induction from axillary shoot explant varied among the six clones tested. Higher number of axillary shoots and shoot regeneration frequency was observed in the clones MH and KA, and the responses to cytokinins and auxins for shoot or root induction were concentration and/or genotype specific. Bhau and Wakhlu (2001) reported variation in callogenetic ability of different explants among three varieties of *Morus alba*. Further, the genotypic differences with respect to callus initiation have been previously observed in *Eucalyptus grandis* $\times$ *E. urophylla* (Barrueto *et al.*, 1999).

Studies on *in vitro* culture in *Sesuvium* have been conducted mostly using a single collected clone, and there have been no reports in testing diverse clones collected from coastal regions differing in environmental conditions. This is the first report on the differential response of various *Sesuvium* clones collected from different coastal regions of India.

Earlier reports of Lokhande *et al.* (2009) on variations in the morphological and genetic diversity analysis and the results of the present study on differential clonal behaviour of *Sesuvium* to shoot multiplication on a optimized shoot multiplication medium suggest that specific *Sesuvium* clone with its capability to tolerate the conditions can be used in the coastal areas for the reclamation of saline soil, restoration of wetlands and phytoremediation of heavy metal contaminated soil depending upon the environmental or climatic conditions of that area. For example, MH clone from Maharashtra with the highest number of multiple shoots could be useful as a potential source for utilization and/or restoration in specific ecological zones. Further, tissue culture grown plantlets of *Sesuvium* clones can be used to study the physiological responses to salt tolerance mechanism under *in vitro* conditions and its relation to *ex vitro* conditions.

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