In Vitro Regeneration of Medicinally Important Shrub *Carissa opaca* from Shoot Apices and Nodal Segments

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Abstract. The study was aimed to develop efficient shoot regeneration from *ex vitro* explants of *Carissa opaca*, an imperative medicinal reservoir. Shoot apices and nodal segments were inoculated on MS (Murashige and Skoog) medium containing BAP (6-benzyl amino purine) and Kin (Kinetin) alone and in combination with NAA (naphthalene acetic acid) and GA₃ (Gibberellic acid). Higher concentrations of both cytokinins were found effective for regeneration from both explants. However, gibberellic acid and NAA addition with cytokinin, no persuading results were achieved. The shoot apices were found more effective in *in vitro* regeneration than nodal segments. The protocol can be effectively used for in vitro multiplication of *C. opaca*, genetic transformation, and secondary metabolite production.

Introduction

Carissa opaca is a perennial shrub comprising of enormous medicinal potential. There are no reports available on the micropropagation of this medicinally important specie. However, reckless collection and deforestation have made this specie near to be encompassed in extinct species. There is an unprecedented need for the reliable source of this plant material for commercial manipulation either by exploiting modern or conventional plant tissue culture techniques. *C. Opaca* is locally known as “Kaurunda” is an evergreen shrub characterized by thorny twigs with alternate leaves. It is found on relatively high altitude as in Pakistan Kashmir, Murree, Abbottabad and Margalla Hills are habitat for this plant. The plant is effective in hepatitis and jaundice [1], antipyretic and anticancer effect [2]. Beside these cardioprotective, restorative potency of reproductive hormones, hepatoprotective activity and anti-inflammatory activities are associated with the fruits and leaves while roots are found anti-oxidative in nature [3].

Several plants fail to produce flower or seed and germinate under the influence of some specific conditions imposed by the climate. This *in vitro* regeneration technique ensures steady supply of plants by making use of least space and time [4]. The advantages of *In vitro* micro propagation is secondary metabolites production, production of genetically engineered plants, controlled environmental conditions according to specific plant needs, threatened plant species conservation, production of clones and identification, year round plant availability, cryopreservation ensuring the preservation of genetic material and greater multiplication rate and others [5]. The objective of the present quest was to develop protocol for the *in vitro* shoot regeneration of *Carissa opaca* from explants.

Materials and Methods

The shoots and nodal explants of *Carissa opaca* collected from the vicinity of Quaid-i-Azam University, Islamabad Pakistan were inoculated on MS (Murashige and Skoog 1962 [6]) basal media supplemented with cytokinin (6-benzyl amino purine; BAP and Kinetin; Kin) alone and with GA₃ (Gibberellic acid) and NAA (naphthalene acetic acid) for shoot organogenesis. Sucrose (30 g/L) was added in MS media as carbon source and pH of the media was adjusted to 5.7. Nobel agar 8 g/L was added and 30 ml media was poured in 100 ml conical flask. The flasks were autoclaved at 121°C and 15 psi for 20 min.

Before inoculation of explants, the explants were disinfected by treatment with 0.1% mercuric chloride solution for 3-4 min and three times washing with autoclaved water. The explants were cut to a size of 0.5 cm in length and were placed on the media under sterile condition. The flasks were kept in growth chamber having 25°C and 16/8 day light cycle with light intensity 10000 lux. After 15 and 30 days of inoculation, percentage of shoot regeneration, shoot length, number of shoots and number of leaves per shoot were noted.

Three explants were inoculated in each flask and three flasks were used for each concentration. Each value indicates mean $\pm$ SD for nine replicates. The means of significantly different values were compared using Duncan's multiple range test (DMRT) at $P < 0.05$.

Results

Shoot tip and nodal segments were inoculated on MS media supplemented with BAP (6-benzyl amino purine) and Kin (Kinetin). Both explants showed 100% regeneration at 4 mg/L BAP and 3 & 4 mg/L Kin. Shoot tips also showed 100% regeneration at 3 & 5 mg/L BAP and 5 mg/L Kin. At lower concentrations of BAP, nodal segments showed better response while shoot tip showed better response at lower concentration of Kin (Fig 1). When both cytokinins were added with GA₃ (Gibberellic acid) or NAA (naphthalene acetic acid), shoot tip explants showed 100 % regeneration rate at most of the combinations. However 100% response from nodal segments was observed only on two combinations of Kin and GA₃ (Fig 2).

Figure 1. Percentage Growth Response of *C. opaca* explants on MS supplemented media with BAP and Kin.

Figure 2. Percentage Growth Response of *C. opaca* explants on MS media supplemented with cytokinin along with GA₃ or NAA.

Table 1 shows that maximum shoot length (1.82 cm) from shoot apices attained at 3 mg/L while maximum number of shoot (2.8) and maximum number of leaves (4.33) were produced at 4 mg/L BAP. Same concentrations of Kin (3 & 4 mg/L) were found best for shoot length, number of shoots and number of leaves, respectively (Table 1). At 4mg/L BAP the shoots protruded from nodal segments attained maximum length (1.17 cm), maximum number of shoots (2.8) and maximum number of leaves (5.6). Nodal segments also showed good shoot length (1.38 cm) and number of leaves (4.4) response at 4 mg/L kin however maximum number of shoots (2) were obtained at 2 mg/L kin (Table 1).

Table 1. Effect of BAP and Kin on shoot regeneration of *C. opaca* from shoot apices and nodal segments.

Hormon (mg/L)	Shoot Apices			Nodal Segment		
	Length	No of	Leaves	Length	No of	Leaves
BAP						
0.5	0.30±0.07f	1.25±0.43	1.25±0.43c	0.27±0.09c	1.25±0.43c	1.50±0.50cd
1	0.55±0.11e	1.75±0.83c	1.50±0.50c	0.63±0.52bc	2.00±0.71b	3.00±1.58b
1.5	0.23±0.05g	1.33±0.47d	2.00±0.00bc	0.15±0.05d	1.67±0.47bc	3.00±0.82b
2	0.75±0.05d	2.50±0.50ab	2.00±0.00bc	0.20±0.00c	2.50±0.50ab	2.00±0.0c
3	1.82±0.74a	1.50±0.76cd	3.50±1.60ab	0.85±0.11b	1.83±0.69bc	3.80±1.12b
4	1.58±0.29ab	2.80±1.17a	4.33±2.13a	1.17±0.14a	2.80±1.17a	5.60±1.50a
5	1.18±0.21c	1.40±0.49cd	2.50±1.71b	1.20±0.10a	1.40±0.49c	3.20±1.60b
Control	1.35±0.05b	2.00±0.0b	1.50±0.50c	0.15±0.05d	2.00±0.0b	1.50±0.50cd
Kin						
0.5	0.40±0.0c	0.50±0.10c	0.50±0.10c	0.50±0.10c	1.00±0.00d	2.00±0.00c
1	0.25±0.05d	0.43±0.05c	0.43±0.05c	0.43±0.05c	1.25±0.43c	2.00±0.00c
1.5	0.20±0.06d	0.10±0.00d	0.10±0.00d	0.10±0.00d	1.60±0.49bc	2.80±0.98b
2	0.35±0.05c	0.60±0.00b	0.60±0.00b	0.60±0.00b	2.00±0.00a	3.00±1.00b
3	1.92±0.20a	1.07±0.35ab	1.07±0.35ab	1.07±0.35ab	1.83±0.37b	4.00±1.15ab
4	1.78±0.29ab	1.38±0.09a	1.38±0.09a	1.38±0.09a	1.80±0.40b	4.40±1.50a
5	1.30±0.24b	1.32±0.26a	1.32±0.26a	1.32±0.26a	1.83±0.37b	4.00±1.15ab
Control	1.35±0.05b	0.15±0.05d	0.15±0.05d	0.15±0.05d	2.00±0.00a	1.50±0.50d

Values are the mean ± standard error (SE) of three replicates per treatment. Each replicate contained four explants. Values followed by different letters indicate significant a difference between means ($P \leq 0.05$) according to one way ANOVA.

Maximum number of shoots (2) from shoot apices were also attained when BAP and Kin (3 & 4 mg/L) were combined with different concentrations of GA₃ (Table 2). However, shoots did not attain better length and maximum was observed in control flasks. The combination of BAP (4 mg/L) or Kin (3 mg/L) with GA₃ (3 or 2 mg/L, respectively) were found better to induce leaves on shoots emerged from shoot apices. Nodal segments also did not show better response in case of shoot length when cultured on BAP and GA₃ combination however 4 mg/L BAP with GA₃ (3 or 4 mg/L) was found better for shoot length and number of leaves. The same response was observed when BAP was replaced with Kin.

Table 2. Effect of cytokinins and GA₃ on shoot regeneration of *C. opaca* from shoot apices and nodal segments.

Hormone (mg/L)		Shoot apices			Nodal Segments		
		Length	No of shoots	Leaves	Length	No of shoots	Leaves
BAP	GA₃						
3	0.5	0.30±0.10e	1.50±0.50bc	1.50±0.50f	0.80±0.10ab	1.00±0.00b	3.00±1.00b
3	1	0.83±0.04c	1.25±0.43c	2.50±0.87e	0.63±0.05b	1.00±0.00b	2.00±0.00c
3	2	0.80±0.22c	2.00±0.00a	7.33±0.94b	0.30±0.10c	1.50±0.50a	2.00±0.00c
3	3	0.35±0.05e	2.00±0.00a	7.00±1.00b	0.40±0.00c	1.00±0.00b	2.00±0.00c
4	0.5	0.85±0.18c	1.75±0.43b	4.00±0.41c	0.45±0.05c	1.00±0.00b	1.50±0.50c
4	1	1.10±0.10b	1.50±0.50bc	3.50±0.50d	0.77±0.12ab	1.33±0.47a	3.00±0.82b
4	2	0.75±0.15cd	2.00±0.00a	6.50±0.50bc	0.45±0.05c	1.00±0.00b	2.00±0.00
4	3	0.73±0.12cd	1.67±0.47bc	8.67±0.94a	0.40±0.10c	1.50±0.50a	5.00±1.00a
Control		1.35±0.05a	2.00±0.00a	1.50±0.50f	1.00±0.60a	1.00±0.60a	1.80±0.40c
Kin	GA₃						
3	0.5	0.30±0.20d	1.00±0.00d	3.00±1.00c	0.70±0.10b	1.00±0.00b	2.50±0.50cd
3	1	0.60±0.08c	1.67±0.47bc	2.00±0.00d	0.60±0.10c	1.00±0.00b	1.50±0.50d
3	2	0.60±0.08c	2.00±0.82a	8.00±1.63a	0.73±0.05b	1.67±0.47a	5.33±1.89a
3	3	0.64±0.27c	1.80±0.40b	6.40±2.33b	0.30±0.00d	1.00±0.00b	4.00±0.00b
4	0.5	1.45±0.05a	2.00±0.00ab	5.00±1.00bc	1.20±0.10a	1.50±0.50a	4.00±0.00b
4	1	1.00±0.20b	1.00±0.00d	2.00±0.00d	0.95±0.05ab	1.50±0.50a	5.00±1.00ab
4	2	0.50±0.16cd	1.67±0.47bc	6.67±2.49b	0.15±0.05e	1.50±0.50a	3.00±1.00c
4	3	0.75±0.15bc	1.50±0.50c	7.00±1.00ab	0.25±0.05d	1.00±0.00b	3.00±1.00c
Control		1.35±0.05a	2.00±0.00ab	1.50±0.50e	1.00±0.60ab	1.00±0.00b	1.80±0.40d

Values are the mean ± standard error (SE) of three replicates per treatment. Each replicate contained four explants. Values followed by different letters indicate significant a difference between means ($P \leq 0.05$) according to one way ANOVA.

Replacement of GA₃ with NAA however in combination with BAP or Kin did not much influences studied parameters. Shoot length did not positively influenced on any combination however number of shoot and number of leaves got better response at different combination or BAP or Kin with NAA (Table 3).

Table 3. Effect of cytokinins and NAA on shoot regeneration of *C. opaca* from shoot apices and nodal segments.

Hormone (mg/L)		Shoot apices			Nodal segment		
		Length	No of	Leaves	Length	No of	Leaves
BAP	NAA						
3	0.5	0.76±0.28bc	1.60±0.49cd	6.00±2.53ab	0.18±0.08d	1.25±0.43bc	1.50±0.50b
3	1	0.98±0.13b	1.75±0.43c	7.50±2.18a	0.22±0.16d	1.80±0.40b	2.60±1.74ab
4	0.5	0.65±0.14c	2.17±0.69a	7.33±2.20a	0.35±0.05c	2.50±0.50a	4.00±0.00a
4	1	0.62±0.16c	1.50±0.50d	4.33±1.80b	0.50±0.21b	1.75±0.43b	4.00±2.00a
Control		1.35±0.15a	2.00±0.00b	1.50±0.50c	1.00±0.60a	1.00±0.00c	1.80±0.40b
Kin	NAA						
3	0.5	0.38±0.19c	1.25±0.43b	5.00±2.24b	0.24±0.10d	1.20±0.40bc	1.80±0.40c
3	1	0.97±0.26b	1.67±0.47ab	6.67±2.40ab	0.47±0.05b	1.33±0.47bc	3.33±0.94bc
4	0.5	0.77±0.23bc	1.50±0.50b	4.50±0.96b	0.35±0.05c	1.50±0.50b	4.00±0.00b
4	1	0.70±0.22bc	1.83±0.37ab	7.50±2.57a	0.47±0.12b	2.33±0.47a	6.00±0.00a
Control		1.35±0.05a	2.00±0.00a	1.50±0.50c	1.00±0.60a	1.00±0.00c	1.80±0.40c

Values are the mean ± standard error (SE) of three replicates per treatment. Each replicate contained four explants. Values followed by different letters indicate significant a difference between means ($P \leq 0.05$) according to one way ANOVA.

Discussion

C. opaca shoot tip and nodal explants showed better regeneration at higher concentration of cytokinin. BAP or kin at 3 mg/L and above showed 100% regeneration potential from shoot tip explants. When BAP and Kin, were combined with GA₃, approximately 100% regeneration response from shoot tip explant was observed excluding few combinations. Replacing GA₃ with NAA also did not favour the regeneration rate where none of the combinations showed 100% response for nodal segments while at few combinations all inoculated shoot tips responded well (100% response). Effectiveness of BAP over Kin for bud break has been reported in a number of plant species [7, 8]. Profound results of BAP over Kin on *in vitro* propagation of *Caralluma tuberculata* and *Cariasa* species have been proven by others [9-11].

Limited numbers of shoot buds were obtained due to vigour of shoot buds and inherent nature of plant. It is evident from results that increasing the concentration of BAP or Kin also triggers increase in the number of shoots and leaves per plant. Such findings have also been reported by others. No substantial changes in growth parameters were observed when explants were treated with cytokinin along with GA₃ and NAA. It is evident from others results that gibberellins did not enhance the shoot length [12]. These results suggest that GA₃ was slightly effective than NAA when used in synergy with BAP or Kin. No significant effect on plant height was observed when GA₃ and NAA were used.

In the node culture, rate of multiplication of *C. opaca* explants is found less than that which was brought about through shoot culture in all treatments of plant growth regulators PGRs (cytokinins, gibberellins and auxins). Shoot cultures have high multiplication rate when cultured at appropriate medium [13, 14]. As *C. opaca* is a shrub, the possible reasons for slow growth, dearth of response to cytokinins, abnormal growth, e.g., hyperhydric transformation, prolonged phenolic exudation, shoot miniaturization, or stunting, shoot necrosis, or excessive callusing hampering shoot multiplication optimization in stage II, have been proposed [15] especially when dealing with woody species.

After 30 days period of shoots establishment, the microshoots were inoculated on full strength and half strength MS basal media. The inoculated shoots were duped into no roots emergence even on augmented MS basal media with IAA and IBA (0.25-2 mg/L). The possible reasons could be the problems like dearth of response (poor or no root induction) to auxin(s), excessive callusing, or deterioration in overall shoot quality for *in vitro* micropropagation as Lynch also proposed these problems for *in vitro* rooting [16].

Conclusions

The present experiments document the very first protocol for *C. opaca* plantlets' production by tissue culture. This study conclude that MS basal media appended with BAP and Kin at 3 and 4 mg/L concentrations was found to be vigorous for shoot regeneration from *ex vitro* explants. However, other synthetic hormones like TDZ or woody plant media can be used to achieve successful rooting and acclimatization. The established *in vitro* propagation procedure for shoot proliferation could be used as a substitute for the propagation of *C. opaca* plantlets.

Conflict of Interest

The authors declare that there is no conflict of interest.

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