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Development of an efficient in vitro propagation protocol for *Satureja punctata* - A rare aromatic and medicinal plant

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ABSTRACT: *Satureja punctata* is an important aromatic and medicinal plant in Lamiaceae family. This plant is threatened mainly due to overgrazing, deforestation, over harvesting of the whole plant for medicinal purpose. This study was aimed to develop an efficient in vitro propagation protocol for *S. punctata* using shoot tips. Shoots excised from in vitro germinated seedlings were used as mother plant for culture induction on MS medium supplemented with different concentrations of 6-benzyl amino purine (BAP) and Kinetin (KIN). Highest shoot induction frequency (100%) with highest mean number (8.25±1.64) of shoot per explant was obtained at 0.5 mg/L BAP. In multiplication media, among different concentrations of BAP, KIN, BAP×KIN and KIN×NAA (α-Naphthalene acetic acid), BAP × NAA found to be an optimum concentration with (26.20±2.71) mean number of shoots. The best shoot height (2.48 ± 0.13 cm) was achieved on growth regulators free medium, which was used as control. Half strength MS medium containing different concentrations of Indole-3- butyric acid (IBA), Indole-3-acetic acid (IAA) and NAA were used for root induction. The highest rooting percentage (100%), root number (5.90±0.48) and root length (1.55±0.11 cm) were attained in the presence of 1.0 mg/L IBA. Up on acclimatization, 97.5% survived plants were recorded. The results demonstrated that, this study was very important for mass propagation and ultimate conservation of this valuable plant.

KEY WORDS: In vitro, medicinal plant, plant growth regulators, *Satureja punctata*

INTRODUCTION

Medicinal and aromatic plants have been recognized since ancient time and they are still used in medicine, food and cosmetic industry (Lahlou, 2004). About 65-80% of the world's population in developing countries mainly depends on medicinal plant for their primary health care (Momtaz and Abdollahi, 2010). In Africa, up to 80% of the population uses traditional medicine for primary health care (WHO, 2003). According to the report of Deribe *et al*. (2006) and Ethiopian Institute of Biodiversity (2007), in Ethiopia around 85% of people use medicinal plants as traditional medicines for their health care.

Satureja species have economic and medicinal importance because of their high essential oil content. With their pleasant fragrance, they are widely used as herbal teas and spices (Satil and Kaya, 2007). Essential oils obtained from the leaves and flowers of different *Satureja* species are commonly used in various industrial applications as flavoring material, perfume and medicine (Teklu *et al*., 1998; Tariku *et al*., 2010). Antibacterial, antifungal, antioxidant, cytotoxic, insecticidal, antidiabetic, anti-leishmanial, insect repellant, hepatoprotective, antiviral, anti-cholinesterase, hypolipidemic-hypoglycemic, anti-inflammatory, anti-nociceptive, nematicidal, anti-proliferative, genotoxic, anti-genotoxic, neuroprotective, ovicidal, anti-biofilm, molluscicidal, antihelmintic, herbicidal, anti-epilepsy, antialzheimer, amoebicidal, nephroprotective, anti-lipase, wound healing, trypanocidal/anti-protozoal, enzyme inhibition, anti-spasmoidal, vasodilatory-vasorelaxant, antitumoral, and diuretic activities of this genus were reported (Tepe, 2015).

Satureja punctata is a flowering plant in Lamiaceae family. It is an erect perennial herb that grows to a height of 10–100 cm (Hedberg *et al*., 2006). In Ethiopia, it grows often on stony slopes and on limestone area or on dry and on rocky ground at an altitude of 600-3840 meters above sea level (Hedberg *et al*., 2006). Teklu *et al*. (1998) isolated 16 chemical components from *S. punctata*. Essential-oil composition, antileishmanial and toxicity study of S. *punctata* from Ethiopia were reported (Tariku *et al*., 2010). Its essential oil showed leishmanicidal effect against *promastigote* that cause leishmaniasis which is a significant and widespread disease among the Ethiopian population (Tariku *et al*., 2010). Essential oils composition of this taxon from Zimbabwe (Chagonda and Chalchat, 2005) was reported. It is used as a medicinal herb to treat bronchitis, stomach ailments and as a febrifuge in different traditional systems in East Africa and in Eritrea (Chagonda and Chalchat, 2005). The aerial parts of this species are used as a traditional medicine to treat headache, stop menstruation, relieve stomach pains, for the treatment of liver diseases, leishmaniasis and improve the quality of milk (Tariku *et al*., 2010; Wolde *et al*., 2010). Essential oils from this species were active

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against selected Gram-positive and Gram-negative bacterial strains (Belay *et al*., 2011). Its antioxidant activity was investigated (Nasser *et al*., 2010).

This species is continuously harvested from its natural habitats. Problems related with its excessive utilization, overgrazing, disruption of the habitat and harvesting before seed set resulted in rare distribution of this valuable medicinal and aromatic plant to use it as cure as well as for industrial purpose. In vitro propagation is very important to germplasm conservation, produce an abundant number of clonal propagules and improve drug-yielding capacity of the plant. However, there is no any reported evidence to date on in vitro propagation as well as classical cultivation of this plant species. Thus, the purpose of this study was to develop micropropagation system for *S. punctata* from shoot tips and this is gives warranty to this rare medicinal plant.

MATERIALS AND METHODS

Plant material

Matured seeds of *Satureja punctata* were collected from Oromia region, Arsi Zone, Amigna district, Sade-Wale Kebele from a particular place called Golja. Seeds were tightly sealed in cotton cloth during transportation to avoid seed loss by water due to its very small size. The seeds were washed with detergent under running tap water and disinfected as they were sealed within cotton cloth. The seeds were surface disinfected with 70% (v/v) alcohol for 8 min and rinsed five times by sterile distilled water, followed by sodium hypochlorite solution containing two drops of tween 20 for 16 min and consequently rinsed three to five times thoroughly with sterile distilled water.

Seeds were planted on 40 mL growth regulators-free MS (Murashige and Skoog, 1962) medium in baby food jars (6 cm diameter) containing 30 g/L sucrose (w/v) and solidified by 8 g/L agar (w/v). Before addition of agar, pH of the medium was adjusted to 5.8 and autoclaved at 121ºC for 15 min under 105 Kpa pressure. Cultures were incubated under light intensity of 22 µmol $m^{-2}s^{-1}$ and 16 h photoperiod provided by white fluorescent lamps at 25±2ºC. Shoots excised from the in vitro germinated seedlings were utilized as stock plant for culture commencements.

Shoots excised from thirty-day-old seedlings were used for culture initiation on MS medium containing BAP (0.0, 0.5, 1.0, 1.5, 2.0 mg/L), Kinetin (0.0, 0.5, 1.0, 1.5, 2.0 mg/L), 30 g/L sucrose (w/v) and 8 g/L agar (w/v). Thirty two shoots per treatment, 8 shoots per culture vessel with 4 replications were examined.

For shoot proliferation, MS medium containing BAP (0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 mg/L), Kinetin (0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 mg/L), BAP in combination with KIN, BAP in combination with NAA, KIN in combination with NAA, 30 g/L sucrose (w/v) and 8 g/L agar (w/v) was used. Thirty shoots per treatment and 10 shoots per culture vessel with 3 replications were used.

Half strength MS medium fortified with 15 g/L sucrose (w/v), 8 g/L agar (w/v), IBA (0.0, 0.25, 0.5, 1.0, 1.5, 2.5 mg/L), IAA (0.0, 0.25, 0.5, 1.0, 1.5, 2.5 mg/L) and NAA (0.0, 0.25, 0.5, 1.0, 1.5, 2.5 mg/L) were utilized for root induction. Magenta jars (7 cm diameter) containing 50 mL medium were used as culture vessels for shoot initiation, proliferation and root induction.

The pH of all media was adjusted to 5.8 before addition of agar and autoclaved at 121ºC with a pressure of 105 Kpa for 15 min. All cultures were kept under light intensity of 22 µmol $m^{-2}s^{-1}$ and 16 h photoperiod provided by white fluorescent lamps at 25±2°C. In this experiment, data were recorded after 4 weeks of culture.

For acclimatization, the rooted shoots were gently washed under running tap water and planted in pots (20 cm diameter) containing sand, garden soil, and compost at a ratio of 2:1:1 respectively. The potted plantlets were covered with polyethylene bags and kept in greenhouse. They were watered as necessary. Ten days later, the polyethylene bags were removed. The survived plantlets were recorded after 30 days of acclimatization.

Experimental design and Statistical Analysis

The design of this experiment was a completely randomized design (CRD). The analysis of variance (ANOVA) was used to differentiate the significant differences among means using statistical data analysis software SPSS version 22.0 at 5% probability level.

RESULTS

Shoot induction

Well induced shoots were observed after 13 days of culture. All initiated shoots appeared to have high quality except those initiated on 1.5 mg/L and 2.0 mg/L BAP, which resulted in vitrified shoots. The highest percentage of shoot initiation (100%) with the maximum mean number of shoot (8.25±1.64) was obtained on a medium fortified with 0.5 mg/L BAP. The next maximum average of shoot (5.55± 1.28) was obtained from a medium containing 2.0 mg/L KIN. The greatest shoot length (2.62±0.17 cm) was recorded on PGRs free-medium (Table 1, Fig. 1).

Shoot multiplication

Influences of BAP and KIN on shoot multiplication

A significant difference was observed among the different concentrations of BAP and KIN in terms of shoot number and shoot length after 4 weeks of culture. The highest mean number of shoot per explant (10.80±2.26)

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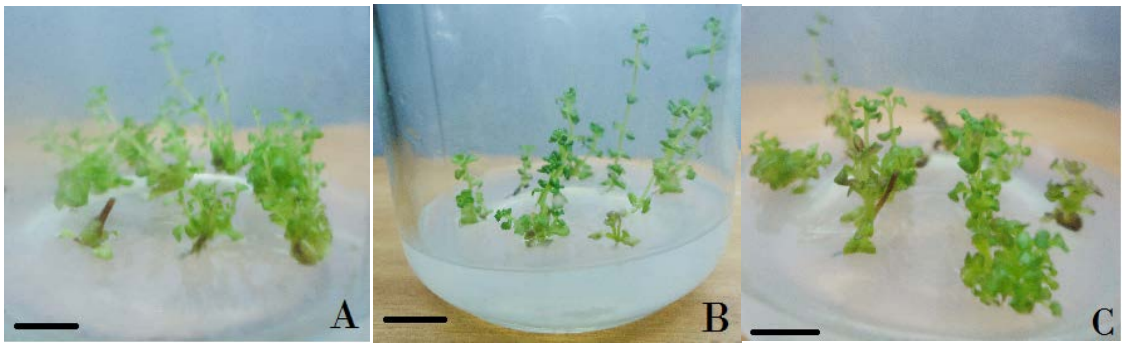


Fig. 1. Shoot induction of *S. punctata* using shoots excised from in vitro germinated seedlings. **A.** 0.5 mg/L BAP, **B.** 0.5 mg/L KIN, **C.** 1.5 mg/L KIN. Bars = 1 cm

Table 1. Effect of BAP and KIN on shoot initiation

Treatment (mgL^{-1}) Kin	BAP	Response (%)	NO. of shoot/explant	Shoot length (cm)
0.0	0.0	100	1.90 ± 0.17^{c}	2.62 ± 0.17^{a}
0.5	0.0	90	3.75 ± 1.18^{bc}	1.72 ± 0.28^{b}
1.0	0.0	85	2.45 ± 0.43^{c}	0.95 ± 0.13^{de}
1.5	0.0	90	3.75 ± 0.80^{bc}	1.05 ± 0.11^{cd}
2.0	0.0	90	5.55 ± 1.28^{b}	1.45 ± 0.20^{bc}
0.0	0.5	100	8.25 ± 1.64^{a}	1.00 ± 0.08^{de}
0.0	1.0	85	1.90 ± 0.29^{c}	0.62 ± 0.08^{de}
0.0	1.5	70	2.40 ± 0.56^{c}	0.60 ± 0.10^{e}
0.0	2.0	70	1.40 ± 0.30^{c}	0.57 ± 0.12^{e}

Means within columns having different letter(s) are significantly different at $p < 0.05$. Data are presented as mean ± SE.

was obtained from medium containing 1.5 mg/L BAP (Table 2). The maximum shoot length (2.48±0.13 cm) was attained on growth regulators free medium, which was used as control (Fig. 2).

Table 2. Effect of BAP and KIN on shoot multiplication from shoot tips of *S. punctata*

Treatment (mgL^{-1}) BAP	KIN	NO. of shoot/explant	Shoot length (cm)
0.0	0.0	2.30 ± 0.29^{d}	2.48 ± 0.13^{a}
0.5	0.0	7.56 ± 2.73^{ac}	0.58 ± 0.10^{cd}
1.0	0.0	5.50 ± 1.02^{bcd}	0.51 ± 0.06^{cd}
1.5	0.0	10.80 ± 2.26^{a}	0.61 ± 0.08^{c}
2.0	0.0	4.20 ± 0.61^{c}	0.66 ± 0.10^{c}
2.5	0.0	8.06 ± 1.64^{ab}	0.53 ± 0.06^{cd}
3.0	0.0	6.73 ± 1.85^{b}	0.36 ± 0.04^{d}
3.5	0.0	1.90 ± 0.62^{d}	0.16 ± 0.04^{d}
4.0	0.0	4.20 ± 0.62^{cd}	0.38 ± 0.05^{d}
0.0	0.5	3.40 ± 0.54^{cd}	1.08 ± 0.16^{b}
0.0	1.0	4.46 ± 0.67^{cd}	1.21 ± 0.24^{b}
0.0	1.5	3.66 ± 0.55^{cd}	0.63 ± 0.06^{c}
0.0	2.0	5.63 ± 0.61^{bd}	0.71 ± 0.10^{c}
0.0	2.5	6.33 ± 0.65^{bc}	0.66 ± 0.15^{c}
0.0	3.0	4.23 ± 0.63^{cd}	0.60 ± 0.09^{cd}
0.0	3.5	3.96 ± 0.32^{cd}	0.58 ± 0.04^{cd}
0.0	4.0	2.73 ± 0.65^{d}	0.31 ± 0.05^{d}

Means within columns having different letter(s) are significantly different at $p < 0.05$. Data are presented as mean ± SE.

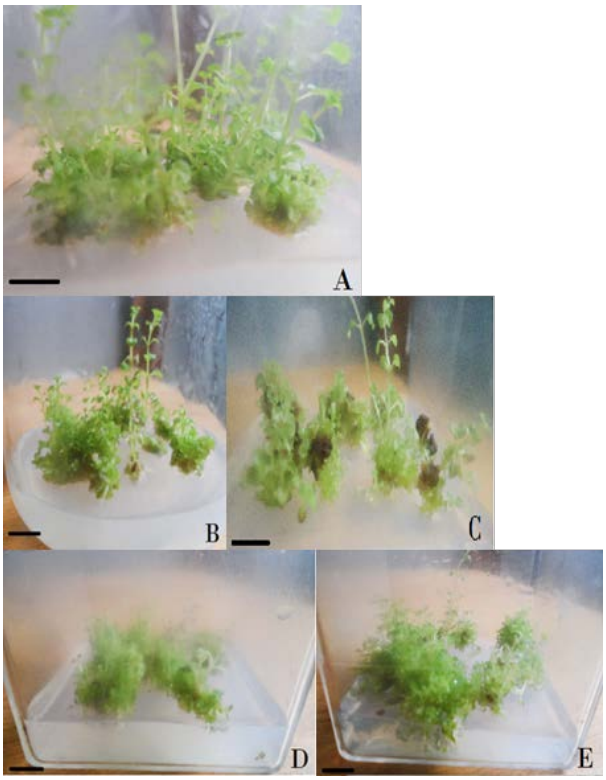


Fig. 2. In vitro propagation of *S. punctata.* **A**. 3.5 mg/L BAP + 0.25 mg/L NAA, **B**. 2.0 mg/L BAP + 0.25 mg/L NAA, **C**. 2.5 mg/L KIN, **D**. 3.5 mg/L BAP + 0.25 mg/L KIN, **E**. 4.0 mg/L BAP + 0.25 mg/L KIN. Bars =2 cm

Synergistic effect of KIN and NAA

Among the various combinations of KIN and NAA used, the MS media containing 0.5 mg/L KIN + 0.25 mg/L NAA and 3.0 mg/L KIN + 0.25 mg/L NAA produced maximum mean number of shoot (2.56±0.26 and 2.06±0.38) respectively. However, these data were not statistically different from shoots (2.30±0.29) achieved on control medium (Table 3). PGRs free-medium resulted in highest mean shoot length (2.48±0.13 cm). Shoots obtained from all these combinations produced spontaneous roots, calli, dwarf shoot and showed poor multiplication rate.

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Table 3. Effect of combination of KIN and NAA on shoot multiplication of shoot tips of *S. punctate.*

Treatment (mgL^{-1}) KIN	NAA	NO. of shoot/explant	Shoot length (cm)
0.0	0.0	2.30 ± 0.29^{a}	2.48 ± 0.13^{a}
0.5	0.25	2.56 ± 0.26^{a}	0.98 ± 0.06^{b}
1.0	0.25	1.96 ± 0.33^{ab}	0.75 ± 0.05^{bc}
1.5	0.25	1.53 ± 0.27^{bc}	0.66 ± 0.11^{c}
2.0	0.25	1.06 ± 0.21^{c}	0.41 ± 0.07^{d}
2.5	0.25	1.03 ± 0.22^{c}	0.53 ± 0.10^{cd}
3.0	0.25	2.06 ± 0.38^{a}	0.45 ± 0.06^{cd}
3.5	0.25	0.40 ± 0.12^{c}	0.20 ± 0.06^{d}
4.0	0.25	0.63 ± 0.13^{c}	0.38 ± 0.07^{d}

Means within columns having different letter(s) are significantly different at $p < 0.05$. Data are presented as mean ± SE.

Table 4. Combined effects of BAP and NAA on shoot multiplication of *S. punctate.*

Treatment (mgL^{-1}) BAP	NAA	NO. of shoot/explant	Shoot length (cm)
0.0	0.0	2.30 ± 0.29^{d}	2.48 ± 0.13^{a}
0.5	0.25	4.13 ± 0.53^{cd}	0.53 ± 0.04^{cd}
1.0	0.25	6.56 ± 1.25^{bc}	0.61 ± 0.05^{c}
1.5	0.25	7.30 ± 0.94^{b}	0.70 ± 0.07^{c}
2.0	0.25	4.10 ± 0.59^{c}	0.71 ± 0.09^{c}
2.5	0.25	1.20 ± 0.25^{d}	0.16 ± 0.03^{d}
3.0	0.25	2.26 ± 0.43^{d}	0.51 ± 0.07^{cd}
3.5	0.25	26.20 ± 2.71^{a}	1.08 ± 0.05^{b}
4.0	0.25	1.93 ± 0.32^{d}	0.36 ± 0.04^{d}

Means within columns having different letter(s) are significantly different at $p < 0.05$. Data are presented as mean ± SE.

Synergistic effect of BAP and NAA

In the presence of 3.5 mg/L BAP + 0.25 mg/L NAA, highest mean number (26.20±2.71) of shoot per explant was produced. This was the significant combination overall treatment tested for multiplication (Table 4, Fig. 2). Best mean shoot length (2.48±0.13 cm) was recorded from PGRs free-medium. Calli were observed on shoots that were cultured on medium containing 1.5 mg/L BAP + 0.25 mg/L NAA, 3.0 mg/L BAP + 0.25 mg/L NAA and 4.0 mg/L BAP + 0.25 mg/L NAA whereas both roots and calli were observed on shoots that were cultured on medium containing 1.0 mg/L BAP + 0.25 mg/L NAA, 2.0 mg/L BAP + 0.25 mg/L NAA and 2.5 mg/L BAP + 0.25 mg/L NAA.

Synergistic effect of BAP and KIN

The highest mean numbers of shoots per explant (18.60 ± 1.85 and 18.10 ± 1.43) were obtained on media augmented with 3.5 mg/L BAP + 0.25 mg/L KIN and 4.0 mg/L BAP + 0.25 mg/L KIN respectively. The highest mean length of shoots (2.48 ± 0.13 cm) was obtained from shoots cultured on growth regulators free medium. Cultures under these combinations were more vigorous and better in shoot proliferation rate as compared to BAP and KIN alone (Table 5, Fig. 2).

Table 5. Combined effects of BAP and KIN on shoot multiplication of *S. punctate.*

Treatment (mgL^{-1}) BAP	KIN	NO. of shoot/explant	Shoot length (cm)
0.0	0.0	2.40 ± 0.42^{ef}	2.48 ± 0.13^{a}
0.5	0.25	4.00 ± 0.54^{e}	1.75 ± 0.28^{b}
0.5	0.5	6.70 ± 0.56^{de}	1.16 ± 0.09c
0.5	1.0	4.50 ± 0.63^{e}	1.71 ± 0.35^{b}
1.0	0.25	4.80 ± 0.52^{e}	1.17 ± 0.12^{c}
1.0	0.5	5.50 ± 0.50^{e}	1.22 ± 0.12^{c}
1.0	1.0	9.55 ± 1.16^{cd}	0.50 ± 0.00^{d}
1.5	0.25	14.75 ± 2.22^{bc}	0.50 ± 0.00^{d}
1.5	0.5	7.60 ± 0.59^{d}	0.98 ± 0.06^{c}
1.5	1.0	7.35 ± 0.73^{d}	0.78 ± 0.05^{d}
2.0	0.25	8.25 ± 0.71^{d}	1.16 ± 0.07^{c}
2.0	0.5	2.30 ± 0.34^{f}	0.52 ± 0.04^{d}
2.0	1.0	0.50 ± 0.18^{f}	0.30 ± 0.09^{d}
2.5	0.25	11.05 ± 1.32^{c}	1.11 ± 0.05^{c}
2.5	0.5	11.25 ± 1.29^{c}	1.02 ± 0.08^{c}
2.5	1.0	11.65 ± 1.57^{c}	1.06 ± 0.08^{c}
3.0	0.25	11.05 ± 1.09^{c}	0.93 ± 0.04^{c}
3.0	0.5	9.35 ± 1.03^{cd}	0.93 ± 0.05^{c}
3.0	1.0	16.20 ± 2.46^{ab}	0.60 ± 0.04^{d}
3.5	0.25	18.60 ± 1.85^{a}	0.75 ± 0.04^{d}
3.5	0.5	15.85 ± 2.07^{ab}	1.02 ± 0.06^{c}
3.5	1.0	17.40 ± 1.62^{ab}	1.02 ± 0.06^{c}
4.0	0.25	18.10 ± 1.43^{a}	0.88 ± 0.05^{c}
4.0	0.5	0.90 ± 0.23^{f}	0.30 ± 0.05^{d}
4.0	1.0	0.55 ± 0.11^{f}	0.27 ± 0.05^{d}

Means within columns having different letter(s) are significantly different at $p < 0.05$. Data are presented as mean ± SE.

Root induction

Medium containing different concentrations of IBA, IAA and NAA showed a significant difference in all parameters. After 4 weeks of culture, MS medium supplemented with 1.0 mg/L IBA resulted in highest mean number of roots per shoot (5.90±0.48) with 100% root formation frequency. The next highest root number per shoot (5.60±0.71) was obtained on the medium containing 0.5 mg/L IBA with 100% root induction response. The MS media containing 1.5 mg/L and 2.5 mg/L IAA produced compact calli. Callus was induced at all concentrations of NAA except at 0.25 mg/L (Table 6). The highest root lengths (1.55±0.11 cm, 1.45±0.11 cm) were obtained on medium containing 1.0 mg/L and 0.5 mg/L IBA respectively (Fig. 3).

Acclimatization

Four weeks later, after planting in greenhouse, the best survival frequency (97.5%) of plantlets was recorded. Plantlets with many branches survived better than plantlets with a single branch (Fig. 4).

DISCUSSION

Shoot induction

Results of the analysis of variance revealed that different concentrations of BAP and KIN had a highly significant effect on shoot initiation percentage, number of

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Table 6. Root induction efficiency of *S. punctata* shoots cultured on MS medium containing IBA, IAA and NAA after 4 weeks of culture.

Treatment (mgL^{-1})	Frequency of root formation (%)	NO. of root/explant	Root length (cm)	NO. of shoot/explant	Shoot length (cm)
IBA					
0.0	75	1.70 ± 0.30^{ef}	0.50 ± 0.08^{c}	3.85 ± 0.78^{a}	2.02 ± 0.30^{b}
0.25	75	3.55 ± 0.81^{cd}	0.80 ± 0.11^{b}	3.95 ± 0.65^{a}	1.05 ± 0.11^{cb}
0.5	100	5.60 ± 0.71^{ab}	1.45 ± 0.11^{a}	2.55 ± 0.42^{b}	2.77 ± 0.23^{a}
1.0	100	5.90 ± 0.48^{a}	1.55 ± 0.11^{a}	4.15 ± 0.59^{a}	1.22 ± 0.20^{cd}
1.5	70	4.05 ± 0.89^{c}	0.53 ± 0.05^{c}	1.95 ± 0.41^{bc}	0.77 ± 0.14^{d}
2.5	55	2.25 ± 0.65^{df}	0.27 ± 0.05^{d}	2.10 ± 0.41^{bd}	1.02 ± 0.17^{cd}
NAA					
0.25	90	4.25 ± 0.49^{bc}	0.45 ± 0.03^{c}	2.30 ± 0.38^{b}	1.17 ± 0.24^{cd}
0.5	00	0.00^{g}	0.00^{e}	0.00^{d}	0.00^{e}
1	00	0.00^{g}	0.00^{e}	0.00^{d}	0.00^{e}
1.5	00	0.00^{g}	0.00^{e}	0.00^{d}	0.00^{e}
2.5	00	0.00^{g}	0.00^{e}	0.00^{d}	0.00^{e}
IAA					
0.25	75	4.20 ± 0.68^{bd}	0.37 ± 0.04^{cd}	2.00 ± 0.35^{bd}	1.32 ± 0.23^{c}
0.5	85	3.35 ± 0.53^{ce}	0.42 ± 0.04^{c}	2.55 ± 0.50^{b}	1.42 ± 0.62^{c}
1	40	2.15 ± 0.70^{de}	0.22 ± 0.06^{d}	1.30 ± 0.24^{cd}	0.77 ± 0.14^{d}
1.5	00	0.00^{g}	0.00^{e}	1.05 ± 0.31^{cd}	0.67 ± 0.22^{d}
2.5	00	0.00^{g}	0.00^{e}	1.30 ± 0.34^{cd}	0.32 ± 0.07^{de}

Means within columns having different letter(s) are significantly different at $p < 0.05$. Data are presented as mean ± SE.



Fig. 3. In vitro rooting of *S. punctata*. **A**. 1.0 mg/L IBA, **B**. 0.25 mg/L NAA, **C**. 0.25 mg/L IAA. Bars = 2 cm.

shoot and shoot length at $p < 0.05$. The shoot induction efficiency of BAP was better than KIN in this taxon. From this result, BAP was more powerful in the stimulation of new bud development. Similar results were reported on different plants including *Sida*



Fig. 4. Ex vitro acclimatization of *S. punctata*. A. 10-day-old, B. 20- day-old, C. 30- day-old. Bars =2 cm.

cordifolia (Sivanesan and Jeong, 2007), *Podophyllum hexandrum* (Chakraborty *et al*., 2010), *Satureja abyssinica* (Teshome and Soromessa, 2015), *Glinus lotoides* (Teshome and Feyissa, 2015b), *Hybanthus enneaspermus* (Velayutham *et al*., 2012).

Shoot induction efficiency of this plant was declined as the concentration of BAP increased. This indicated that, elevated concentration has negative effect on shoot formation and lower concentration must be used. Comparable outcomes were reported on

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Ricinus communis (Alam *et al*., 2010), *Cordeauxia edulis* (Seyoum and Mekbib, 2014), *S. abyssinica* (Teshome and Soromessa, 2015), *G. lotoides* (Teshome and Feyissa, 2015b). However, at higher concentration of KIN, the mean number of shoots per explant increased. This is in agreement with Teshome and Soromessa (2015) on *S. abyssinica.* Shoot length of this plant was reduced as the amount of cytokinin increased. This indicates the influence of cytokinins on reduction of apical dominance and encourages axilary and adventitious shoot formation. Seyoum and Mekbib (2014) on *C. edulis*, Teshome and Feyissa (2015b) on *G. lotoides* and Teshome and Soromessa (2015) on *S. abyssinica* reported similar results.

Vitrified cultures were induced at highest concentration of BAP and this is parallel with previous report on *S. abyssinica* (Teshome and Soromessa, 2015).

Shoot multiplication

Different concentrations of BAP in combination with KIN, BAP and KIN alone and each of them in combination with NAA were used for shoot proliferation. BAP was found to be the most suitable cytokinin for mass micropropagation of *S. punctata*. Multiple shoot production capability of BAP was also reported in *G. lotoides* (Teshome and Feyissa, 2015a, 2015b), *Thymus sipyleus* (Baba and Yurekli, 2000), *Thymus mastichina* (Fraternale *et al*., 2003), *Thymus satureioides* (Aicha *et al*., 2013). However, KIN was superior over BAP in propagation of *S. abyssinica* (Teshome and Soromessa, 2015). The present finding revealed that, an average of multiple shoot was declined with increased concentration of cytokinins. Further increasing the level of cytokinin beyond the optimum concentration was not tolerated in this plant species. Analogous results were reported on *G. lotoides* (Teshome and Feyissa, 2015a, 2015b), *S. abyssinica* (Teshome and Soromessa, 2015). In this investigation, the height of shoot was declined from control medium to higher contraction of BAP and KIN, which is in agreement with the findings on *G. lotoides* (Teshome and Feyissa, 2015a, 2015b).

The response of plant to different hormone type and concentration is not the same. The negative interaction of KIN × NAA was relatively resulted in less number of shoot than KIN alone. Mean number of shoot under this combination was not significantly different from the mean number of shoots on PGRs free medium. The mean shoot number in some of the combinations was lower than on control medium and this is in agreement with Teshome and Soromessa (2015) report on *S. abyssinica*. Spontaneous root and callus formation were observed almost on all media supplemented with KIN and NAA combination. This might be due to the presence of NAA. Similar results were also reported on *Physalis minima* (Farhana *et al*., 2009; Sheeba *et al*., 2010; Mungole *et al*., 2011), *Physalis peruviana* (Otroshy *et al*., 2013), *S. abyssinica* (Teshome and Soromessa, 2015). However, a positive interaction effect of BAP×NAA in which the maximum mean number of shoots was produced than entirely over the treatment used for multiplication. This revealed the efficiency of BAP in multiple shoot formation than KIN when combined with NAA. BAP×NAA was found to be effective in many plant species (Ndoye *et al*., 2003; Ahmad *et al*., 2002). Spontaneous rooting and callus formation were observed on some media fortified with BAP×NAA. Several authors observed that NAA as effective PGRs for callus induction (Rehman *et al*., 2003; Velayutham *et al*., 2006; Velayutham *et al*., 2012).

Root induction

Root establishment and their development highly depend on exogenous auxin. The highest frequency of root formation, mean number and mean length of root, mean number of shoot and shoot length were seen in the presence of IBA. This shows that, IBA was the favorable auxin than NAA and IAA for root initiation in *S. punctata*. This efficiency of IBA was also reported in *G. lotoides* (Teshome and Feyissa, 2015a, 2015b), *S. abyssinica* (Teshome and Soromessa, 2015), *Gymnema sylvestre* (Komalavalli and Rao, 2000), *Gloriosa superba* (Sivakumar and Krishnamurthy, 2000), *Tridax procumbens* (Jesmin *et al*., 2013), *Psoralea corylifolia* (Pandey *et al*., 2013), *Chlorophytum borivilianum* (Bera *et al*., 2009), *Stevia rebaudiana* (Jitendra *et al*., 2012), *Origanum sipyleum* (Oluk *et al*., 2009), *Cunila galioides* (Fracaro *et al*., 2001), *Achyranthes aspera* (Sen *et al*., 2014), *Vitex agnus-castus* (Balaraju *et al*., 2008), *Operculina turpethum* (Sebastinraj *et al*., 2013). But super-optimum concentration of auxin (IBA, NAA and IAA) reduced root number. This agrees with Rafique *et al.* (2012) in *Dendrobium sabin*, Ali *et al.* (2012) in *Dalbergia sissoo*, Jesmin *et al*. (2013) in *T. procumbens.*

Even though IAA was better than NAA, in both there was poor root induction and in some of their concentrations no root at all. Teshome and Soromessa (2015) in *S. abyssinica* and Bohidar *et al*. (2008) in *Ruta graveolens* reported the same outcomes. However, NAA was better in *Lippia filifolia* (Peixoto *et al*., 2006), *Clitoria ternatea* (Rout, 2004) and *T. satureioides* (Aicha *et al*., 2013). On another hand, Echeverrigaray *et al*. (2010) reported the effectiveness of IAA over IBA and NAA.

CONCLUSION

The depletion of wild population can be prevented using biotechnological tools such as micropropagation for enhancing yield, commercial utilization, genetic

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improvement, produce identical propagules and germplasm preservation. BAP×NAA and IBA were found to be the most appropriate hormone for shoot proliferation and root induction respectively. Thus, this study provides an effective protocol for *S. punctata* to generate plant material throughout the year.

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