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Full Length Research Paper

In vitro micro-propagation of endangered ornamental plant-*Neotchihatchewia isatidea* (Boiss.) Rauschert

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The ornamental plant, *Neotchihatchewia isatidea*, is an endangered species of Turkey and threatened by complete extinction in the future. Therefore, *in vitro* multiplication of this species can be valuable for commercial production and germplasm conservation. Immature embryos of *N. isatidea* were cultured for initiation on Murashige and Skoog medium (MS) supplemented with N^6-benzylamino-purine (BAP) and α-naphthaleneacetic acid (NAA). Shoot primordia were visible within 5 - 6 weeks and the shoot primordia later developed into normal shoots 10 - 12 weeks after the culture initiation on calli developed from immature embryos. Shoot tips were also excised from developed plantlets for direct shoot organogenesis and cultured on MS shoot induction medium supplemented with BAP (0.5, 1.0 and 2.0 mg/l), kinetin (KIN) (0.5, 1.0 and 2.0 mg/l) and thidiazuron (TDZ) (0.05, 0.10 and 0.50 mg/l). Direct multiple shoots from shoot tips developed in most media tested. High shoot multiplication (3.73), high rooting (53 %) number of root per shoot (3.66) and survival ratio (46.6 %) were achieved.

Key words: *Neotchihatchewia isatidea,* shoot regeneration, immature embryo, shoot tip, rooting.

INTRODUCTION

The flora of Turkey is very rich in terms of wild medicinal, aromatic and ornamental plants. There are the great numbers of ornamental plants of bulbous-tuberous, woody and herbaceous perennials and biennials in Turkish flora. The important geophytes species mostly belong to *Liliaceae, Iridaceae* ve *Amaryllidaceae* family. However, there are another species belonging to other families with attractive flowers and have pleasant smell in Turkish flora *Neotchihatchewia isatidea* (Boiss.) Rauschert (= Syn: T*chihatchewia isatidea* Boiss.) belonging to *Brassicaceae* family is also a monotypic and the genus is perennial and biennial herb with their attractive flower colours, interesting indumentum and nice smell (Mutlu and Dönmez, 2003). This genus is also an endangered species of Turkey and threatened by complete extinction in the future. Medium level propagation rate in nature and irregular collection of *N. isatidea* plants from their habitat also hampers the cultivation of the species in future.

Tissue culture technique is powerful tool which can be employed as an alternative to the conventional method of vegetative propagation with the objective of enhancing the rate of multiplication of desired genotypes (Hussey, 1986a; Hussey, 1986b; Murashige, 1990; Naik and Nayak, 2005). Micropropagation allows the production of pathogen-free plants with uniform quality for agronomic cultures and for germplasm conservation. *In vitro* culture also enables plants to produce secondary metabolites under controlled culture conditions. Furthermore, the establishment of cell culture of this plant may considerable potential in the future as an alternative for the production of new secondary metabolites. *In vitro* shoot regeneration and micro-propagation of *N. isatidea* has

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Abbreviations: MS, Murashige and Skoog medium; BAP, N^6-benzylamino-purine; NAA, α-naphthaleneacetic acid; KIN, Kinetin, IBA, indole-3-butyric acid; IAA, indole-3-acetic acid; and TDZ, thidiazuron.

not been reported previously up to date. Therefore, the objective of the current study was to develop, for the first time, a reliable and prolific shoot multiplication procedure using immature embryo explants of *N. isatidea* as a starting material. Development of high shoot multiplication system for *N. isatidea* will help efficient micro-propagation and germplams conservation of the species in the future.

MATERIALS AND METHODS

Plant material and fruit surface sterilization

Seeds of *N. isatidea* were collected from wild flora of Erzurum province of Turkey and sown in the experimental fields of Department of Field Crops, Faculty of Agriculture, University of Ankara, Turkey and the plants were flowered at the second growing year (Figure 1a). Immature fruits of *N. isatidea* and later mature seeds were harvested in July and August months respectively two years later after sowing. The fruits were surface-sterilized in 50% comercial bleach (Axion) for 10 min and then rinsed three times with sterilized water. Each fruit contains 1 or 2 seeds. The seeds were also surface sterilized for 2 min in 70% (v/v) ethanol and then for 30 min in 50% commercial bleach (Axion) for germination studies.

Isolation of immature embryos

Immature seeds of surface sterilized fruits were removed. The seed coat encasing the immature embryo was peeled away and the seed was squeezed hard using scalpel handle until the immature zygotic embryo (approximately 0.8 - 1.0 mm in length) was on the loose as described by Mirici et al. (2005).

Culture conditions

Basal media salts, vitamins, sucrose, agar and growth regulators were obtained from Sigma Chem. The pH of medium was adjusted to 5.7 with 1 N NaOH or 1 N HCl before autoclaving at 121ºC, 1.4 kg/cm^2 for 20 min. All cultures were kept at 24 ± 1ºC under cool white fluorescent light (35 µmol m^{-2} s^{-1}) with 16-h photoperiod. Growth regulators thidiazuron (TDZ), N^6-benzylamino-purine (BAP), Kinetin (KIN), indole-3-butyric acid (IBA), indole-3-acetic acid (IAA) and α-naphthaleneacetic acid (NAA) were filter-sterilized using a Milipore filter (0.22 µm pore size) and added to the autoclaved medium under aseptic conditions after cooling to 45°C. Stock solution of 1 mg/ml TDZ was prepared either by using dimethyl sulphoxide (DMSO; Sigma Technical Information bulletin 1996) or 50% ethanol as solvent. Ethanol diluted TDZ was incorporated into the medium and retained its high activity even after autoclaving as described by Khawar et al. (2004).

Callus and shoot induction from immature embryo

Immature embryos were transferred to shoot induction on MS callus and shoot induction medium supplemented with different combinations and concentrations of BAP (1.0, 2.0 and 4.0 mg/l) and NAA (0.5 and 1.0 mg/l), 3% (w/v) sucrose and 0.7% (w/v) agar in Petri dishes(10 x 100 mm) containing 35 ml of medium.

Shoot proliferation from shoot tips

Shoot tips (3 - 4 mm length, 2 - 3 mm width) were also excised from developed plantlets for direct shoot organogenesis and the explants were cultured on MS shoot proliferation medium supplemented with BAP (0.5, 1.0 and 2.0 mg/l) KIN (0.5, 1.0 and 2.0 mg/l) and TDZ (0.05, 0.1 and 0.5 mg/l). A medium without plant growth regulators was also used as control. The explants were sub-cultured several times on the same media in Magenta (GA-7) vessels at 3 or 4 weeks interval until prolific shoot formation. Scoring for adventitious shoot regeneration and micro-propagation was done after 8 - 10 weeks of culture.

Rooting of regenerated shoots and acclimatization of the plantlets

The regenerated shoots (about 1.5 cm) from explants were excised and individually transferred to ten different rooting medium consisting of half strength MS basal medium supplemented with 20 g/L sucrose and various concentrations of IBA, IAA and NAA in Magenta vessels. After 52 - 59 days in rooting medium, the rooted shoots were removed from the culture medium and the roots were washed in sterile distilled water to remove all traces of agar. Then, the rooting ratio, the number of roots per shoot, were recorded and mean root lengths were determined after six weeks of culture. Rooted plantlets were acclimatized in a growth chamber and at 90% humidity and transferred to 16 cm pots containing 1:1 mixture of soil and turf and grown till maturity under green house conditions. Plantlets were irrigated as needed.

Experimental design and statistically analysis

Each treatment had four replicates consisting of Magenta vessels, each containing 5 explants for micro-propagation. The micro-propagation experiments were repeated at least two times and all results were pooled. Each treatment had six replicates consisting of Magenta vessels, each containing 5 explants for rooting study. Significance was determined by analysis of variance (ANOVA) and the differences between the means were compared by Duncan's multiple range test using MSTAT-C computer programme (Michigan State University). Data given in percentages were subjected to arcsine (√X) transformation (Snedecor and Cochran, 1967) before statistical analysis.

RESULTS AND DISCUSSION

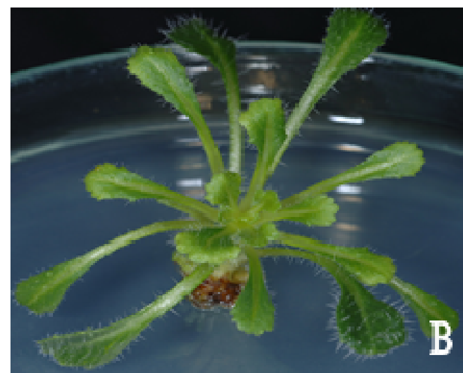
In order to increase germination ratio and to obtain disinfected ex-plant source, the surface sterilized seeds of *N. isatidea* were placed on half-strength MS medium containing 3% sucrose and 0.8% agar. Other surface sterilized seeds were put between two filter papers (Type MN 751) placed in Petri dishes (100 x 10 mm) moistened with -7.5 ml sterile water. However, the germination ratio was 0% on both half-strength MS medium and moistened sterile filter papers.

Mature or immature embryos which have high regeneration capacity may be alternative ex-plant source for the micro-propagation. Moreover, immature embryos can be exquisite source of ex-plant to overcome ex-plant contamination. Therefore, the immature embryo explants used were cultured for shoot multiplication. Any contamination was not observed on the embryo explants in initial establishment. Immature zygotic embryos have healthy appearance within three or four weeks of culture. After five or six weeks in culture, most of the immature embryo

explants used were completely covered with compact calli. All of the embryo explants had a compact basal callus, which was excised and discarded with following sub-cultures. After 10-12 weeks in culture, shoot regeneration was visible on MS media containing BAP and NAA on some embryo explants. One zygotic shoot was also observed on embryo explants and this shoot was immediately cut from the base of shoot. Shoot primordia were visible and the shoot primordia later developed into normal shoots 10-12 weeks after the culture initiation. Well-developed shoots (approximately 2-3 cm length) were observed after 5 - 6 months in culture Regeneration frequency was very low (no data shown) These "unbranched" and "unrooted" shoots were transferred to new culture vessels for further plantlet development. The shoots were grown and rooted on MS medium.

Shoot tips were also excised from developed plantlet for direct shoot organogenesis studies. The shoot tips were cultured on MS shoot induction medium supplemented with BAP (0.5, 1.0 and 2.0 mg/l) KIN (0.5, 1.0 and 2.0 mg/l) and TDZ (0.05, 0.1 and 0.5 mg/l). Shoot proliferation from shoot tips was visible after 4 weeks in most media tested. All explants developed shoots after 9-10 weeks of culture initiation in all concentrations of BAP, TDZ and KIN. BAP stimulated the production of auxiliary shoots regardless of concentration (Figure 1b). BAP also induced significantly more auxiliary shoots when compared to concentration of KIN and TDZ. The number of shoots per ex-plant and mean shoot length were significantly influenced by plant growth regulators ($p < 0.01$). The highest number of shoots per ex-plant was obtained from a MS medium supplemented with 1.0 and 2.0 mg/l BAP. Outstanding increases in shoot multiplication were also observed on a medium containing 1.0 and 2.0 mg/l BAP in shoot tips. Considering both percenttage of explants producing shoots and the mean length of shoots, the best shoot multiplication was also achieved on a range of media containing 1.0 and 2.0 mg/l BAP.

Treatments of *N. isatidea* shoot explants with 2.0 mg/l BAP promoted significantly more shoots than did the control and other cytokinins, but also stimulated massive callus formation on most of the explants. Therefore, higher concentrations of BAP were not recommended for shoot proliferation in this genus as callus interferes with following rooting and acclimatization. Avoiding callus formation minimizes risk of somaclonal variation (Larkin and Scowcroft, 1981). High BAP levels (1.0 or 2.0 mg/l) also caused the shoots to turn yellowish with some vitrification on the shoots as reported in previous study (Constantine, 1986). However, the morphological response of explants to BAP, KIN and TDZ treatments differed markedly and BAP produced thicker, more laterally branched and longer shoots than TDZ and KIN. At the concentrations tested for the growth regulators, TDZ was the least effective for shoot proliferation and caused considerably vitrification on the shoots. Moreover, tested



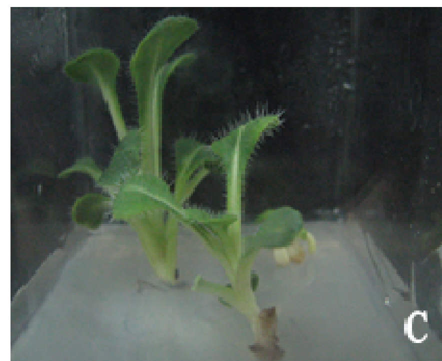


Figure 1. *In vitro* micro-propagation of *N. isatidea*. (A) *N. isatidea* plants with flowers at the experimental fields. (B) Prolific shoot formation from shoop tips after 12 weeks culture on a MS medium containing 2.0 mg/l BAP. (C) Rooting of regenerated shoot tips on half strength MS medium supplemented with 0.25 mg/l IBA. (D) Acclimatization of rooted plantlets in the pots containing 1:1 mixture of soil and turf.

Table 1. *In vitro* clonal propagation of *N. isatidea* from shoot tips on MS media containing various concentrations of TDZ, KIN and BAP.

TDZ	KIN	BAP	Callus**	*Number of shoots per ex-plant*	*Mean shoot length (cm)*
-	-	-	-	0.26 d*	1.2 d*
0.05			+	0.41 d	0.8 e
0.10			+	0.33 d	1.0 de
0.50			+	0.23 d	0.2 f
		0.50	-	2.93 b	3.2 b
		1.00	+	3.73 a	4.4 a
		2.00	+	3.53 a	4.2 a
	0.50		-	0.97 c	3.1 b
	1.00		-	2.76 b	2.1 c
	2.00		+	2.83 b	2.3 c

*Means within a column followed by the same letter are not significantly different by Duncan's multiple range test ($p < 0.05$).
**Callus present (+) or absent (-).

Table 2. Effect of IAA, IBA and NAA concentrations on rooting of *in vitro* regenerated shoots after 6 weeks of rooting treatment.

Growth regulators (mg/l)		Callus**	Rooting (%)	Number of root per shoot
IAA	0.10	+	26.6 bc*	1.0 e*
	0.25	+	26.6 bc	2.77 b
	0.50	+	13.3 c	0.33 f
IBA	0.10	-	33.3 b	1.77 c
	0.25	-	53.3 a	3.66 a
	0.50	+	33.3.b	1.77 c
NAA	0.10	-	26.6 bc	1.33 d
	0.25	-	13.3 c	0.76 ef
	0.50	+	13.3 c	1.33 d

*Values within a column followed by different letters are significantly different at the 0.01 probability level using Duncan's multiple range test.
**Callus present (+) or absent (-).

concentrations of TDZ were more callogenic than those of BAP and KIN (No data shown).

The longest shoots were produced on a MS medium containing 2.0 mg/l BAP. Increasing of BAP (1.0 or 2.0 mg/l) and KIN (1.0 or 2.0 mg/l) on MS media encouraged the shoot length. However, TDZ decreased mean shoot length and similarly, TDZ has been reported to inhibit shoot elongation in several woody species in previous studies (Huetteman and Preece, 1993; Lu, 1993).

In vitro clonal propagation of many plant species through tissue cultures has been frequently based on the prosperous adjustment of the type and combination of plant growth hormones (Murashige, 1990; Tran, 1981; Gürel and Gürel, 1996; Uranbey et al., 2005). Moreover, selection of a suitable ex-plant at correct developmental stage plays a key role in the successful establishment of culture under *in vitro* conditions. The culture medium with specific growth regulator concentrations influenced the organogenesis in the present study and shoot tips could be used for rapid clonal propagation with optimized culture medium and BAP also played a major and distinctive role in the induction of shoot multiplication by organogenesis. (Tables 1 and 2).

N. isatidea may need improved root system against cold and drought conditions in their natural habitat. Adventitious root formation is very important for the vegetative propagation and a key step in micro-propagation systems. An efficient rooting modifications may cause high frequency of rooted shoots and a high-quality root system. Regenerated shoot tips (30 - 50 mm length) were excised and rooted readily in half strength MS medium supplemented with different concentrations (0.1, 0.25 and 0.50 mg/l) of IBA, NAA and IAA to induce the development of roots and to reduce duration of root induction (Figure 1c). Rooting was observed from the cut ends of the shoots between 42 - 56 days in most media tested. The percentage of rooting and the number of roots per shoot have changed significantly with different concentrations of NAA, IBA and IAA ($p < 0.01$). When considering both rooting ratio and the number of roots per shoot, the

best rooting was achieved on half strength MS medium containing 0.25 mg/l IBA. Moreover, among the different auxins tested, 0.25 mg/l IBA was considered to be the best, because of the formation of thick main roots. High concentrations of NAA, IBA and IAA did not strongly stimulate root formation; however, callus formation was strongly stimulated by high concentrations of all auxin types. High concentration of IAA (0.50 mg/l) severely decreased number of root per shoot and induced callus formation on base of the root. Well rooted shoots were rinsed with sterilized water to remove residual rooting media and were transplanted to pots containing 1:1 mixture of soil and turf, and grown in a growth chamber. The survival rate of regenerated plantlets transferred to soil was the highest (46.6%) following root initiation (Figure 1d). All tissue culture-derived plants grew well, showed no morphological variations when compared with seed derived plants. It could be concluded that IBA had more stimulatory side effects than that of NAA and IAA. Similarly, IBA, as a synthetic auxin, was reported to induce adventitious rooting for a wide range of different species (George, 1993) and was preferred based on the results of prior studies (Galiana et al., 1991; Darus, 1993).

In conclusion, the present study describes, for the first time, micro-propagation system for *N. isatidea*. On the basis of our experiments, immature embryo ex-plants seem to be a good starting material for micro-propagation of *N. isatidea.* This protocol also offers a potential system for improvement of this important ornamental plant.

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