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ORIGINAL PAPER

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EFFICIENT *in vitro* PLANT REGENERATION FROM CULTURED LEAF AND PETIOLE EXPLANTS OF *Isatis constricta* Davis

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ABSTRACT

Isatis concstricta Davis (an endemic plant of Turkey) suffers from low propagation rates under natural conditions and is threatened due to fast unplanned urbanisation. The study compared the effects of variants of BA + NAA on shoot regeneration on leaf and petiole explants excised from one week old *in vitro* regenerated seedlings. MS medium containing 1 mg L^{-1} BA + 1 mg L^{-1} NAA induced maximum proliferation on petiole and leaf explants with 13.33 and 12.75 shoots per explant repectively. However, leaf explant induced shoots were sturdy and healthier compared to petiole explant induced shoots. These shoots were rooted on MS medium containing 0.5 mg L^{-1} IBA and the plants were acclimatized in peat moss and sand (v/v). They grew to flowering under *ex vitro* conditions. This system of regeneration is advantageous for conventional propagation and the results will help in establishment of a powerful and meaningful micropropagation system for *I. constricta*.

Key words: direct organogenesis, dye plant, micropropagation, mass proliferation

INTRODUCTION

The genus *Isatis* belonging to *Brassicaceae* family has over 50 species that grow in a range of climatic and soil conditions. Several species are used as dyes or for medicinal purposes. All types of *Isatis* species, are important biennial plants known since Neolithic age and they played an important role in several parts of Europe till late 17th century [Hamburger 2003].

Plant belonging to *Isatis* species are possible sources to obtain antioxidants to deactivate reactive oxygen species (ROS) [Lu and Foo 1995]. The plant seeds are rich in linolenic acid (27.74%), erucic acid (25.5%), oleic acid (16.19%), linoleic acid (10.49%) and aracidic acid (10.22%) [Dolya et al. 1972].

Isatis species has well documented history as medicinal herbs in Europe and in Traditional Chinese Medicine (TCM) since centuries [Mohn 2009]. All of them contain several biologically active environment friendly, compounds like glucobrassicin and indole glucosinolates (GLs), that are reported to have antibacterial, antiviral, antimicrobial, antitumoral, anticarcinogenic and antimutagenic effects. Glucobrassicin and its derivatives have potential antitumoral effect against mammary cancer. It also decrease levels lung carcinogen related urinary metabolites among tobacco smokers [Tang and Eisenbrand 1992, Wu et al. 1997 a, b, Kim and Milner 2005]. Besides this, it is a significantly important source of bioactive

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molecules and indolic compounds that could be exploited as fine chemicals.

It is still popularly used as dye in traditional Turkish marbling industry, since very early times. It is also used as an indigo dye plant in carpet industry of Turkey [Kizil et al. 2007]. The People in England, British and Celt islands extensively prepared woad dye to make inks, paint woods to protect them from decaying and other household purposes.

Isatis constricta is an important member of this group, and is found in upper South East Anatolia and Central Mediterranean region at an altitude of 1200–1300 m [Tubives 2015]. It was very abundant naturally at many locations a few years back and is now under serious threat due to fast increasing unplanned urbanization. Previous studies show a very few literature on *in vitro* micropropagation studies of *Isatis* spp. There is dire need to improve their culture both through traditional agronomic techniques suitable for their cultivation and *in vitro* biotechnological approaches; which will help in biochemical, molecular, biological and enzymatic studies in future.

The study aimed to find and compare impacts of different concentrations of BA + NAA on regeneration of shoots from leaf and petiole excised from of one week old *in vitro* regenerated *I. constricta* seedlings.

MATERIALS AND METHODS

Plant material, surface sterilization and regeneration. The seeds of *Isatis constricta* were collected from Prof. Dr. Suleyman Kizil, and the voucher samples are deposited in the Herbarium of the Department of Field Crops, Faculty of Agriculture, Dicle University, Diyarbakır, Turkey. They were surface sterilised in 25% commercial bleach (Ace 5–6% NaOCl) containing 1/100ml tween-20 for 20 min. Once, the sterilisation was complete, the bleach was decanted carefully and the seeds were rinsed 3 × 5 min in double distilled sterilized water. Thereafter, the seeds were cultured on MS medium [Murashige and Skoog 1962] for 5–7 d to obtain seedlings of desirable size (3–4 cm). The leaf and petiole explants were taken from germinating seedlings under aseptic conditions and cultured on MS basal medium containing 0.5, 1 mg L^{-1} BA + 0.5, 1.0, 2.0 mg L^{-1} NAA (6 combinations – tab. 1) supplemented with 0.65% agar (Duchefa The Netherlands) and 3.0% sucrose.

Each experimental treatment used 32 explants divided into eight replications such that each replication contained 4 explants. pH of all culture media was adjusted to 5.7 ±0.1 using 1 M KOH or 1 M HCl before adding agar. All media was autoclaved at 118 KPa pressure and temperature of 121°C for 20 min. All cultures were incubated under 16 h light photoperiod provided with white fluorescent light (42µMol photons^{-2} s^{-1}) at 24 ±2°C. Well developed shoots were rooted on MS medium containing 0.5 mg L^{-1} IBA.

Acclimatization. After 6 weeks, all rooted plants were aggregated and agar was removed very carefully from roots and the plants were transferred to pots containing sand and peat moss (v/v) in plastic pots covered with polythene bags. These were maintained in growth chamber at 24 ±2°C under light maintaining 12 h light photoperiod. Once the plants began to grow, the transparent polythene bags were removed and they were left for growth and flowering.

Statistical analysis. The experiments data was evaluated using IBM SPSS 20 for Windows. Statistical analysis was performed using univariate analysis after six weeks of culture. Post hoc Duncans multiple range tests were performed for comparison of means. Arcsine transformation was performed for the all data given in percentages before subjecting them to statistical analysis [Snedecor and Cochran 1967].

RESULTS AND DISCUSSION

Adventitous shoot regeneration from leaf and petiole. All of the surface sterilsed seeds using 25% commercial bleach for 20 min showed 100% germination after 6–7 days of culture (fig. 1a). Subsequently, leaves and petioles were isolated from germinating seedlings (fig. 1b) and cultured for regeneration.

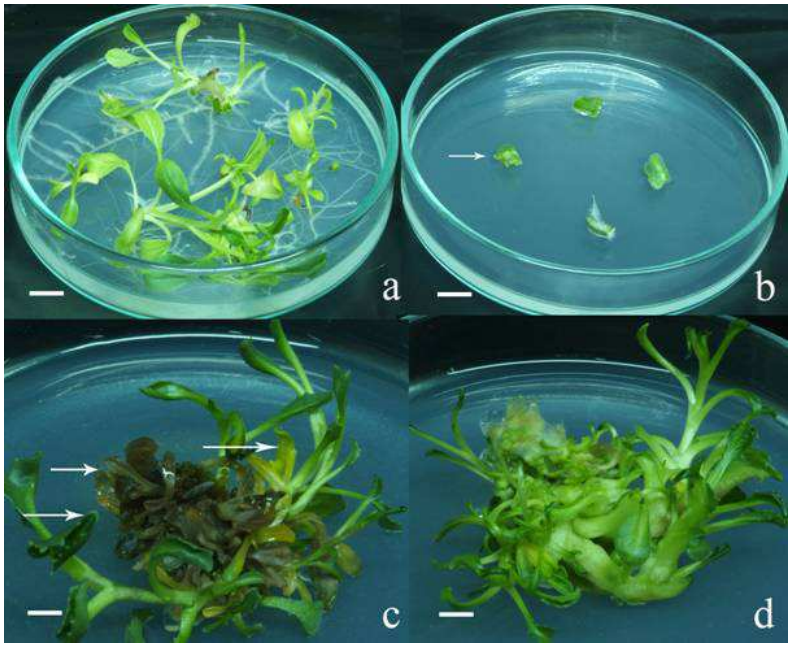

Fig. 1. Effects of various concentrations of BAP + NAA on shoot regeneration from leaf explant of *I. constricta* (a) germination of from seeds after 6–7 days of culture (b) leaf explants taken for regeneration (c) shoot regeneration on MS medium containing 1 mg L^{-1} BAP-mg L1 mg L^{-1} NAA on petiole explant and necrosis on some tissues (d) shoot regeneration on leaf explant on MS medium containing 1 mg L^{-1} BAP-mg L1 mg L^{-1} NAA. Bar Fig. 1a, b = 1.1 cm, Fig. 1c, d = 0.70 cm

Table 1. Effects of various concentrations of BAP-NAA on shoot regeneration from leaf and petiole explant after six weeks of period

Plant growth regulator combinations (mg L^{-1})		Percentage (%) of shoot regeneration		Mean number of shoots per explant		Mean shoot length (cm)	
BAP (mg L^{-1})	NAA (mg L^{-1})	leaf	petiole	leaf	petiole	leaf	petiole
0.5	0.5	66.67abA	75.00abA	7.33bA	3.67cB	0.36dB	0.39bcA
0.5	1.0	100.00aA	100.00aA	10.00aA	8.08abA	0.39dA	0.66abA
0.5	2.0	66.67abB	100.00aA	7.67bA	7.00abA	0.68bcA	0.99aA
1.0	0.5	100.00aA	75.00abB	9.99aA	7.42bB	0.46 cAB	0.66abA
1.0	1.0	100.00Aa	100.00abB	13.33aA	12.75aA	0.85bA	0.47bcB
1.0	2.0	100.00aA	91.667aA	8.67bA	6.42bB	3.03aA	0.40bcB
MS medium (control)		0.00	0.00	0.00	0.00	0.00	0.00

Means of different values shown by same small letters in a column are not statisitically different using Duncans multiple range test at 0.05 level of significance
Means of different values shown by same capital letters in a row are not statisitically different using t test at 0.05 level of significance

Previous studies suggest successful tissue culture of the *Isatis* species using various explants [Peng and Wang 1994, Zhang et al. 2003, 2004, Khawar et al. 2008, Leal et al. 2009, Saglam and Ciftci 2010]. Both explants used in this study showed adventitous shoot regeneration on all variants of BA + NAA with start of swelling after 7th day of culture. Visible shoot initials were induced on the explants after about 13–18 days of culture depending on the concentration and combinations of BA + NAA. Regeneration was carried out on two variants of BAP + 3 variants of NAA (six combinations) such that each concentration of BAP + 3 variants of NAA made one group. The concentration of BA + NAA used in the experiment had variable effects on shoot regeneration from leaf and petiole explant. Percentage of shoot regeneration ranged 66.67–100% and 75–100% on leaf and petiole explants of *I. constricta* respectively (tab. 1). A general comparison of the two explants revealed that maximum shoot regeneration (100%) on leaf explant was achieved on 4 combinations of plant growth regulators (0.5 mg L^{-1} BAP + 1 mg L^{-1} NAA, 1 mg L^{-1} BAP + 0.5 mg L^{-1} NAA, 1 mg L^{-1} BAP + 1 mg L^{-1} NAA and 1 mg L^{-1} BAP + 2 mg L^{-1} NAA); whereas 100% shoot regeneration on petiole was recorded on three combinations of BA + NAA only (0.5 mg L^{-1} BAP + 1 mg L^{-1} NAA, 0.5 mg L^{-1} BAP + 2 mg L^{-1} NAA, 1 mg L^{-1} BAP + 1 mg L^{-1} NAA).

This could also be explained as 0.5 mg L^{-1} BAP + 1 mg L^{-1} NAA (one combination in first sub group) and 1 mg L^{-1} BAP + 0.5, 1 or 2 mg L^{-1} NAA (three combinations in 2nd sub group) were higly suitable for shoot regeneration on leaf explant. Whereas, in case of petiole explant, 0.5 mg L^{-1} BAP + 1 and 2 mg L^{-1} NAA (two combinations in first sub group) and 1 mg L^{-1} BAP + 1 mg L^{-1} NAA (one combination in 2nd sub group) were suitable for 100% shoot regeneration on petiole explant. No shoot regeneration occured on MS medium (control). The results are in agreement with the experimental results of Peng and Wang [1994] and Zhang et al. [2003, 2004] and Khawar et al. [2008]. All of them agree that plant growth regulator combinations, genotype, seedling age and root induction medium behave variably in shoot regeneration on *Isatis* species. Zhang et al. [2006] promoted orthogonal design to study callus induction and differentiation from cotyledons and hypocotyls of *I. tinctoria.* Their results indicated interaction between BA and 2,4-D induced significant effect on callus induction from hypocotyls. They found interaction between BA and NAA with significant effect on callus induction from cotyledons. Based on direct analysis they suggested that it was easier to induce calli on MS medium containing BA + NAA.

A comparison of the behaviour of explants further showed variations in terms of number of shoots per explant and shoot length (tab. 1). Mean number of shoots per leaf and petiole explant ranged 7.33–13.33 and 3.67 to 12.75 using *I. constricta* respectively. Both explants induced maximum number of shoots on MS medium containing 1 mg L^{-1} BAP + 1 mg L^{-1} NAA (fig. 1c, d). Whereas, no shoot per explant was recorded on MS medium in each case. All shoots on leaf explant were healthy and vigorous; whereas, the shoots induced on petiole explant induced hyperhydricity based necrosis variably (fig. 1c). It was noted that maximum number of shoots per explant (among 2 subgroups of BAP + NAA used for regeneration), was recorded when the MS medium contained either 0.5 mg L^{-1} BA + 1 mg L^{-1} NAA or 1 mg L^{-1} BA + 1 mg L^{-1} NAA. The other two combinations of BA + NAA with in each sub group were variably inhibitory to induce shoots on both leaf and petiole explant. This showed that concentration of NAA in the culture medium significantly affected induction of shoots. Leal et al. [2009] noted 3.4 shoots per explant obtained on B_5 medium supplemented with 1.0 mg L^{-1} BA. They noticed that addition of auxin to regeneration media failed to multiply the shoots.

A variation in the shoot length was also very noticeable based on type of explant, the concentration and combination of plant growth regulators. Shoots that regenerated on petiole explant never elongated beyond 0.99 cm; however, the shoots regenerated on leaf explant achieved maximum shoot length of 3.03 cm on MS medium containing 1 mg L^{-1} BA + 2 mg L^{-1} NAA (tab. 1). Leaf explants based shoot regeneration was more vigorous compared to regeneration on petiole explants (fig. 1d). The shoot length on leaf and petiole explants ranged 0.36–3.03 cm and

0.39–0.99 cm respectively. The results show that length of shoots was significantly affected both by the concentrations of plant growth regulators in regeneration media and type of explant on *I. constricta*. The shoot regenerated on MS medium containing 0.5 mg L^{-1} BAP + 0.5, 1, 2 mg L^{-1} NAA on leaf and petiole explant showed increase in shoot length with each increase in the concentration of NAA in the culture medium. Whereas, the shoots regenerated on MS medium containing 1 mg L^{-1} BAP + 0.5,1, 2 mg L^{-1} NAA showed increase and decrease in shoot length with each increase in the concentration of NAA on leaf and petiole explant respectively. Leal et al. [2009] found that *I. tinctoria* nodal segments of young plants cultured in MS and Gamborg (B_5) medium supplemented with BA or KIN, with or without NAA induced variable length of shoots. The researchers find significant effect of adding activated charcoal on shoot length and regenerated internodes on MS medium containing 1.0 mg L^{-1} KIN. Saglam and Ciftci [2010] also regenerated leaf and hypocotyl explants of *I. tinctoria* on MS medium solidified with 6 g L^{-1} agar and 15 g L^{-1} isubgol containing different concentrations of BA + NAA. They found maximum shoot regeneration of 12.65 and 17.80 shoot per leaf explant on agar and isubgol gelled MS medium containing 1.00 mg L^{-1} BA + 0.25 mg L^{-1} NAA and 0.50 mg L^{-1} BA respectively. Maximum shoot regeneration of 19.87 and 20.55 shoots per hypocotyl explant on agar and isubgol gelled medium was recorded on MS medium containing 0.50 mg L^{-1} BA + 0.25 mg L^{-1} NAA and 1.00 mg L^{-1} BAP, respectively.

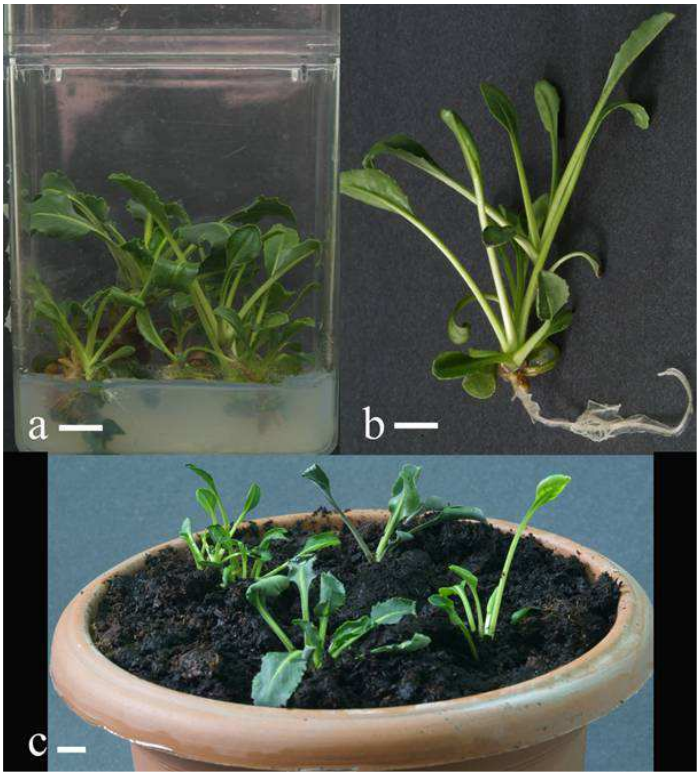

Fig. 2. Rooting and acclimatisation of *I. constricta* (a) rooting of well developed shoots regenerated on MS medium containing 0.5 mg L^{-1} IBA in pots. Bar Fig. 2a, b = 1 cm, Fig. 1c = 2 cm

Fig. 3. Potted acclimatized plants after eight weeks of culture. Bar = 3 cm

The longest shoots of 3.03 cm noted on leaf explant of *I. constricta*; Zhang et al. [2004] found that 22.2 µM or more BA concentration induced negative effects on shoots. It was observed that elongation *I. constricta* shoots was affcted by the type of explant, plant growth regulators and their combinations. This is in agreement with Peng and Wang [1994] who studied diploid *I. indigotica* hypcotyl explants. Peng and Wang [1994], Zhang et al. [2004] observed that tetraploid *I. indigotica* hipocotyl and cotyledon explants needed MS medium for regeneration. They noted that MS medium was more suitable compared to Gamborg B_5 and White medium.

The longest leaf explant regenerated shoots on MS medium containing 1 mg L^{-1} BA + 2 mg L^{-1} NAA were rooted on MS medium containing 0.5 mg L^{-1} IBA (fig. 2 a, b).

The rooted plants were not difficult to acclimatize in the growth room. The plants were transferred to pots after 8–10 weeks and showed fast acclimatisation (fig. 3) in the growth chamber. In confirmation to previous studies, the regenerated plants had no problem in acclimatisation. Saglam and Ciftci [2010] rooted *I. tinctoria* on MS medium containing 0.75 mg L^{-1} IBA. Growth of roots & shoots was very visible during acclimatisation on both plant species. Regenerated plants were comparable with normal plants but were more vigorous. They bloomed and set seeds; without showing any sign of stress during growth. Survival rate of the acclimatized plants was 100%. The time required for completing the cycle of the *in vitro* shoot regeneration, rooting and acclimatization was 20 weeks. The study reports variable behaviour of rooting of *I. constricta* that could be rooted on 0.5 mg L^{-1} IBA; Contrarily, Zhang et al. [2004] rooted *I. indigotica* on ½ ×MS medium containing 0.1 mg L^{-1} IBA. Peng and Wang [1994] used NAA for rooting of shoots regenerated on MS medium containing diploid *I. indigotica* hypocotyl explants. Variations in results could be due to different explants and plant species used in the two experiments.

CONCLUSIONS

It is concluded that the results of this research will contribute positively to objective of the study and constitutes a powerful method to micropropagate *I. constricta* meaningfully.

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