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PLANT REGENERATION FROM *ONOBRYCHIS SUBNITENS* BORNM. HYPOCOTYL EXPLANTS VIA SOMATIC EMBRYOGENESIS AND ORGANOGENESIS

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An efficient procedure is established here for plant regeneration from hypocotyl explants in *Onobrychis subnitens* Bornm., an important forage legume in Iran. Two types of calli, embryogenic and non-embryogenic, were induced from hypocotyl explants on MS medium supplemented with 2,4-D and BAP at different concentrations. To initiate morphogenesis, embryogenic calli were transferred to MS medium with 0.5 $mg \cdot l^{-1}$ NAA and 1 $mg \cdot l^{-1}$ BAP. Initiated somatic embryos or adventitious buds developed into plantlets following culture on MS medium without any growth regulators or with 2 $mg \cdot l^{-1}$ NAA, respectively. All the regenerated plants were normal with respect to morphology and growth characters.

Key words: Hypocotyl culture, *Onobrychis subnitens* Bornm., organogenesis, plant regeneration, somatic embryogenesis.

INTRODUCTION

Onobrychis Miller (Fabaceae), with nearly 170 species, is distributed mainly in north temperate regions, but centers of its diversity are in the eastern Mediterranean and western Asia. Some taxa of the genus are cultivated as fodder or as ornamental plants (Ranjbar et. al., 2004; Ranjbar et. al., 2007; Ranjbar, 2008). *Onobrychis subnitens* Bornm. with a large range of habit types, is an interesting perennial species, endemic to Iran and widely grown as a pasture legume.

Legumes have been regarded as recalcitrant to transformation and their in vitro regeneration is highly genotype-dependent (Somers et al., 2003). Regeneration via organogenesis or embryogenesis is the basis of tissue culture methods, and without regeneration it is impossible to produce transgenic plants. Regeneration through somatic embryogenesis results in a low frequency of chimera and a high number of regenerants (Neves et al., 1999). These characteristics render somatic embryogenesis an attractive system for the introduction of genomic traits of interest by genetic engineering (Neves et al., 1999). Although there have been many reports of in vitro plant regeneration of forage legumes such as *Medicago* (Nolan et al., 1989; Denchev et al., 1991; Li and Demarly, 1996; Neves et al., 1999), *Trifolium* (Maheswaran and Williams, 1986; Radionenko et al., 1994; Rybczynski, 1997), *Onobrychis viciaefolia* (Gu, 1987; Lees, 1988; Pupilli et al., 1989, Yang and Nakashima, 1992; Sancak, 1999), *Astragalus adsurgens* (Luo and Jia, 1998) and *Astragalus melitoides* (Tian and Xi, 1989; Zhang et. al., 1994; Hou and Jia, 2004), there has been no report to date on tissue culture of *Onobrychis subnitens*. Here we report somatic embryogenesis and organogenesis of whole plants from hypocotyl-derived calli of *O. subnitens* for the first time.

MATERIALS AND METHODS

Seeds of *Onobrychis subnitens* were collected in a wild population from the Kurdistan province of Iran. They were immersed in 70% (v/v) ethanol for 1 min, surface-sterilized in 5% calcium hypochlorite solution for 15 min and rinsed three times with sterile distilled water. The seeds germinated aseptically

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Abbreviations: ANOVA – Analysis of variance; BAP – 6-benzylaminopurine; 2,4-D – 2,4-dichlorophenoxy acetic acid; IAA – indole-3-acetic acid; IBA – indole-3-butyric acid; MS – Murashige and Skoog (1962) medium; NAA – α-naphthaleneacetic acid.

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in growth-regulator-free MS (Murashige and Skoog, 1962) medium solidified with 0.8% (w/v) agar at 25±2°C under a 16 h photoperiod (light intensity 50 μmol m^{-2} s^{-1}). Hypocotyl and cotyledon segments measuring 5-8 mm were dissected from 7-day-old seedlings and used as explants. They were cultured in MS medium supplemented with 2,4-D and BAP in different combinations (Tab. 1) and maintained at 25±2°C in darkness for callus initiation. All cultures were carried out in Petri dishes (6 cm diam) containing 20 ml medium and covered with parafilm. The pH of all media was adjusted to 5.7–5.8 before sterilization by autoclaving at 121°C for 15 min. To initiate morphogenesis of *Onobrychis subnitens*, calli were transferred to 150 ml glass Erlenmeyer flasks containing 40 ml of MS medium with different concentrations of NAA and BAP at 25±2°C under a 16 h photoperiod with 50 μmol m^{-2} s^{-1} irradiance provided by cool-white fluorescent tubes. Data on the frequency of callus-producing somatic embryos and adventitious buds were recorded after 4 weeks (Tab. 2). Twenty pieces of calli were used for each experiment, done in triplicate. Data were analyzed using SAS ver 6.12 (SAS Institute, 1995, Cary, N. C.). ANOVA was used to test statistical significance, and the significance of differences among means was tested with Duncan's (1955) multiple range test. Somatic embryos were transferred to MS medium without growth regulators for further development. Adventitious buds were subcultured on MS medium with 0.1 mg·l^{-1} IAA and 1 mg·l^{-1} BAP for 2 weeks to enhance elongation and then transferred to MS medium with 2 mg·l^{-1} NAA for rooting. The rooted plantlets developed from somatic embryos or adventitious buds were subsequently transplanted to plastic pots containing an autoclaved 3:1 mixture of sand and soil. Pots were covered with polyethylene bags for 2 weeks and kept at 25±2°C under a 16 h photoperiod (50 μmol m^{-2} s^{-1}, cool-white fluorescent light). Later they were gradually exposed to low humidity by intermittent removal of the cover and were transferred to a shaded greenhouse.

To track the histological development of *O. subnitens*, calli with somatic embryos were fixed in FAA (formalin:glacial acetic acid:ethanol, 5:5:90, v/v/v) for 24 h, dehydrated in a graded ethanol series and then embedded in paraffin wax. Embedded tissues were cut 10 μm thick with a rotary microtome. Sections were stained with hematoxylin and observed under a light microscope.

TABLE 1. Effect of concentration of 2,4-D and BAP (mg·l^{1}) on embryogenic callus induction in *Onobrychis subnitens* after 3 weeks

Growth regulator (mg·l^{-1})	Explants producing somatic embryos* (%)
2,4-D (0) + BAP (0)	0.0
2,4-D (0.01) + BAP (0.01)	0.0
2,4-D (0.01) + BAP (0.1)	0.0
2,4-D (0.01) + BAP (1)	12.6 ± 1.6 bc
2,4-D (0.1) + BAP (0.01)	1.4 ± 0.3 d
2,4-D (0.1) + BAP (0.1)	26.7 ± 2.1 b
2,4-D (0.1) + BAP (1)	36.3 ± 5.7 b
2,4-D (1) + BAP (0.01)	49.3 ± 1.3 ab
2,4-D (1) + BAP (0.1)	60.9 ± 3.1 a
2,4-D (1) + BAP (1)	64.5 ± 3.2 a
2,4-D (1) + BAP (2)	53.5 ± 3.4 ab
2,4-D (2) + BAP (2)	51.8 ± 1.8 ab

*data within a column followed by different letters differ significantly at $p < 0.05$ by Duncan's multiple range test.

TABLE 2. Effect of concentration of NAA and BAP (mg·l^{-1}) on somatic embryogenesis and organogenesis in *Onobrychis subnitens* after 4 weeks

Growth regulator (mg·l^{-1})	Calli producing somatic embryos* (%)	Somatic embryos producing plantlets* (%)	Calli producing adventitious buds* (%)
NAA (0) + BAP (0)	0.0	0.0	0.0
NAA (0) + BAP (0.5)	0.0	0.0	0.0
NAA (0) + BAP (1)	0.0	0.0	21.8 ± 1.2 e
NAA (0.1) + BAP (0)	0.0	0.0	0.0
NAA (0.1) + BAP (0.5)	26.4 ± 2.5 c	18.5 ± 1.2 d	38.0 ± 3.6 c
NAA (0.1) + BAP (1)	39.2 ± 2.3 bc	26.7 ± 3.6 c	67.1 ± 5.6 a
NAA (0.5) + BAP (0)	7.7 ± 2.1 d	6.1 ± 2.3 d	0.0
NAA (0.5) + BAP (0.5)	80.4 ± 4.1 a	71.5 ± 4.6 a	30.9 ± 2.3 cd
NAA (0.5) + BAP (1)	87.9 ± 1.4 a	79.6 ± 3.8 a	34.3 ± 3.2 c
NAA (0.5) + BAP (2)	48.2 ± 2.1 b	34.6 ± 4.0 b	52.2 ± 4.4 b

*data within a column followed by different letters differ significantly at $p < 0.05$ by Duncan's multiple range test.

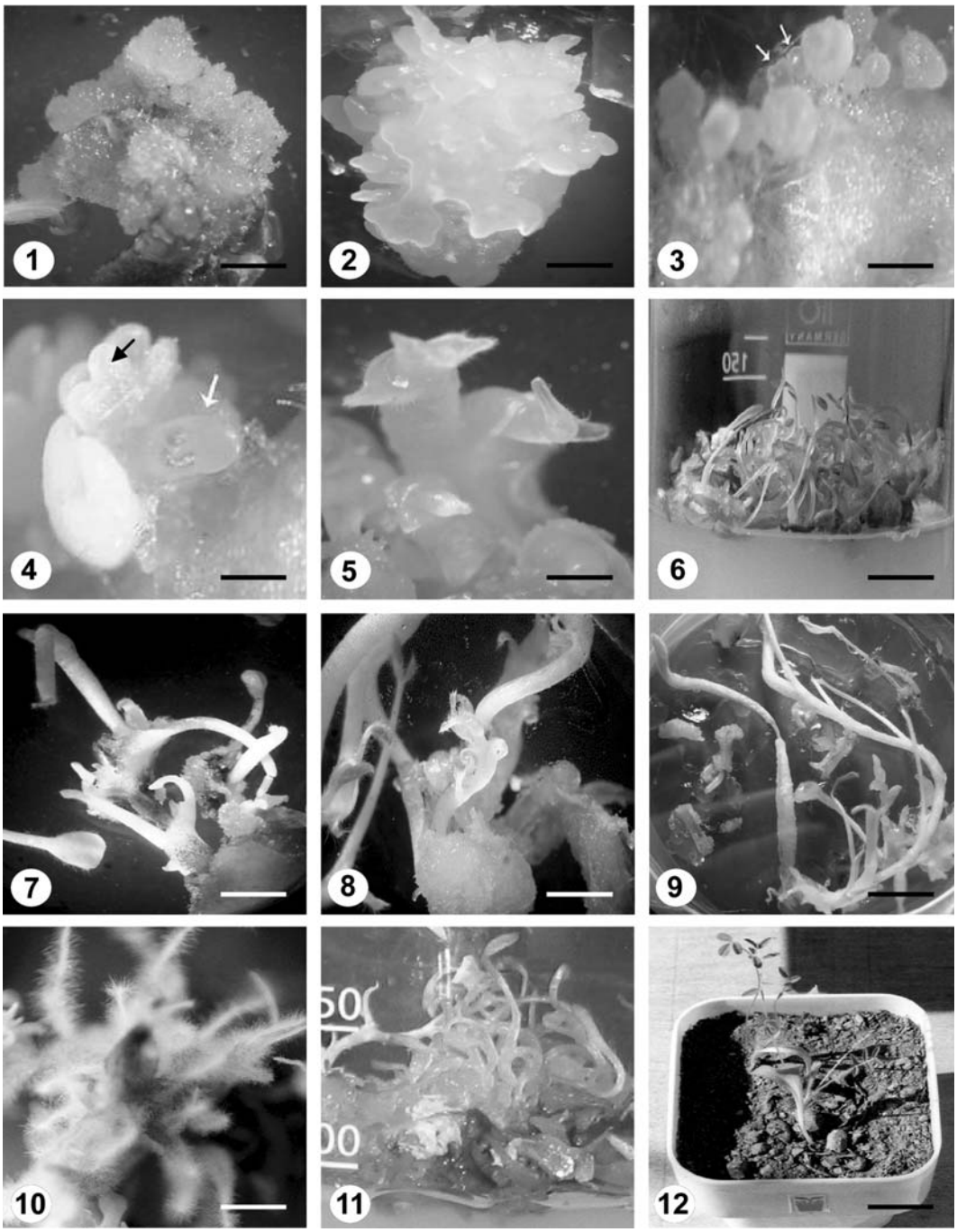
RESULTS

The hypocotyl explants swelled and initiated calli within 2–3 weeks after culture. The calli obtained in all treatments could be differentiated into two types by color and texture. Type I calli were friable and yellowish, with an uneven surface or bearing small granular structures on the surface, and the growth rate was high. Type II calli were soft and yellow-brown, grew slowly, and had a few masses with no morphogenetic potential (Figs. 1,2). Type I calli were induced only from hypocotyl explants. The optimum level of 2,4-D and BAP at which the maximum frequency (64.5%) of embryogenic callus formation was obtained was 1 $mg \cdot l^{-1}$ (Tab. 1). The percentage of embryogenic callus induction declined at a concentration of 2,4-D and BAP higher than 1 $mg \cdot l^{-1}$. Upon transfer of the hypocotyl-derived embryogenic calli (Type I) to MS medium with 0–0.5 $mg \cdot l^{-1}$ NAA and 0–2 $mg \cdot l^{-1}$ BAP, somatic embryos and adventitious buds were induced simultaneously following 4 weeks of culture (Tab. 2). Somatic embryos were induced only from Type I calli. The highest frequency of embryoids (87.9%) was obtained with 0.5 $mg \cdot l^{-1}$ NAA and 1 $mg \cdot l^{-1}$ BAP. The somatic embryos passed through each of the typical developmental stages (heart-shaped, torpedo and cotyledonary), and about 80% of them converted to plantlets in growth-regulator-free MS medium after 4 weeks (Figs. 3–6, 13–15). Organogenetic buds were induced from Type I calli on MS medium with 0–0.5 $mg \cdot l^{-1}$ NAA and 0.5–2 $mg \cdot l^{-1}$ BAP. The maximum adventitious bud frequency (67.1%) was obtained with 0.1 NAA and 1 $mg \cdot l^{-1}$ BAP (Fig. 7). Adventitious buds were significantly improved following culture on MS medium with 0.1 $mg \cdot l^{-1}$ IAA and 1 $mg \cdot l^{-1}$ BAP for 2 weeks (Figs. 8, 9). The elongated shoots were detached and transferred to MS medium containing 2 $mg \cdot l^{-1}$ NAA for rooting (Figs. 10, 11). Nearly all of the shoots differentiated roots within 2 weeks in this medium. After 2 weeks of acclimatization, plantlets developed from somatic embryos and adventitious buds were transferred to the greenhouse (Fig. 12). All regenerated plants were normal with respect to morphology and growth characters.

DISCUSSION

Many factors including the choice of growth regulators and choice of explants are responsible for successful somatic embryogenesis (Luo et. al., 1999). Induction of embryogenic callus was usually promoted by increasing 2,4-D and BAP concentrations to 1 $mg \cdot l^{-1}$ in *Onobrychis subnitens* (Tab. 1). The percentage of embryogenic callus induction declined at 2,4-D and BAP concentration higher than 1 $mg \cdot l^{-1}$. The optimum concentrations of 2,4-D and BAP we found for somatic embryo induction are not completely consistent with other reports from work with some legumes. A high 2,4-D level (up to 2 $mg \cdot l^{-1}$) in combination with a low BAP level (up to 0.5 $mg \cdot l^{-1}$) in the callus induction medium was reported necessary to enhance embryogenic callus production in some legumes (Nagarajan et. al., 1986; Nolan et al., 1989; Luo et. al., 1999). Although somatic embryogenesis has been observed in many plants from various explants (Williams and Maheswaran, 1986), the choice of explants is crucial for inducing embryogenic callus in *Onobrychis subnitens*. Embryogenic callus was produced only from hypocotyl segments on the media tested, supporting the conclusion that the internal state of explant cells is of prime importance in the expression of somatic embryogenesis, with other conditions such as exogenous growth regulators simply enabling expression of this intrinsically determined pattern of development (Zimmerman, 1993; Williams and Maheswaran, 1986). Embryoid production was observed on MS medium with 0.5 $mg \cdot l^{-1}$ NAA in combination with 1 $mg \cdot l^{-1}$ BAP. The somatic embryo developed and converted to plantlets in growth-regulator-free MS medium. There are many reports that embryo development was usually associated with reduction or omission of auxin from the medium (Ammirato, 1987; Carman, 1990). In our work, adventitious buds were significantly improved following culture on MS medium with 0.1 $mg \cdot l^{-1}$ IAA and 1 $mg \cdot l^{-1}$ BAP for 2 weeks. This result is similar to that reported for another legume species, *Astragalus melilotoides*, in MS medium supplemented with 0.25 $mg \cdot l^{-1}$ IAA and 0.5 $mg \cdot l^{-1}$ BAP (Hou and Jia, 2004). The elongated shoots differentiated roots within 2 weeks by transfer of detached shoots to MS medium containing 2 $mg \cdot l^{-1}$ NAA. IBA has been reported to be more favorable for root induction than NAA in some other legumes such as *Astragalus melilotoides* (Hou and Jia, 2004).

This is the first reported protocol for plant regeneration from hypocotyl-derived calli of *Onobrychis subnitens* by somatic embryogenesis and organogenesis. Hypocotyl-derived somatic embryos are desirable explants because they can produce genetically stable plantlets and therefore are suitable for genetic transformation (Bespalhok and Hattori, 1998; Hou and Jia, 2004). The same results were reported for *Onobrychis viciaefolia* and *Astragalus melilotoides* (Hou and Jia, 2004).



Figs. 1–12. Somatic embryogenesis and organogenesis in *Onobrychis subnitens*. **Fig. 1.** Non-embryogenic callus induced from hypocotyl explants on MS medium with 0.01 mg·l^{-1} 2,4-D, 0.1 mg·l^{-1} BAP and 30 g·l^{-1} sucrose after 3 weeks. Bar = 2 mm. **Fig. 2.** Embryogenic callus induced from hypocotyl explants on MS medium with 1 mg·l^{-1} 2,4-D and 1 mg·l^{-1} BAP and 30 g·l^{-1} sucrose after 3 weeks. Bar = 2 mm. **Fig. 3.** Globular somatic embryos induced on MS medium 0.5 mg·l^{-1} NAA and 1 mg·l^{-1} BAP and 30 g·l^{-1} sucrose after 4 weeks (arrows). Bar = 1 mm. **Fig. 4.** Heart-shaped and torpedo somatic embryos developed in growth-regulator-free MS medium (arrows). Bar = 1 mm. **Fig. 5.** Somatic embryos at cotyledonary stage. Bar = 1 mm. **Fig. 6.** Regenerated plantlets from somatic embryos developed in growth-regulator-free MS medium after 4 weeks. Bar = 10 mm. **Fig. 7.** Adventitious buds and shoots induced from hypocotyl-derived callus on MS medium with 0.1 mg·l^{-1} NAA, 1 mg·l^{-1} BAP and 30 g·l^{-1} sucrose after 4 weeks. Bar = 10 mm. **Figs. 8,9.** Shoot elongation in MS medium with 0.1 mg·l^{-1} IAA, 1 mg·l^{-1} BAP and 30 g·l^{-1} sucrose after 2 weeks. Bar = 5 mm. **Fig. 10.** Rooting of regenerated shoots on MS medium with 2 mg·l^{-1} NAA after 2 weeks. Bar = 5 mm. **Fig. 11.** Plantlets obtained by rooting of regenerated shoots after 2 weeks. Bar = 10 mm. **Fig. 12.** Potted plant. Bar = 15 mm.

Figs. 13–15. Histological sections of somatic embryogenesis in *Onobrychis subnitens*. **Fig. 13**. Globular somatic embryo. Bar = 500 μm. **Fig. 14.** Heart-shaped somatic embryo. Bar = 500 μm. **Fig. 15**. Cotyledonary somatic embryo. Bar = 500 μm.

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