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Micropropagation protocol for the hybrid sorrel *Rumex tianschanicus* × *Rumex patientia*, an energy plant. Histological, SEM and flow cytometric analyses

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ABSTRACT

Rumex tianschanicus x Rumex patientia is a prospective plant as a source of renewable energy and is also an important medicinal plant. In the present experiments an efficient and reproducible method for plant regeneration through the direct induction of shoot buds from hypocotyls was developed. The highest frequency of organogenetic response of explants was obtained on 7- and 12-day-old hypocotyls that had been cultured on the regeneration medium MS + 0.17 mg/l IAA + 2.2 mg/l BAP + 2% sucrose (44.06% and 48.88%, respectively). Successfully rooted plantlets were acclimated to *in vivo* conditions with more than a 90% survival rate. Histological analysis revealed direct organogenesis. Shoot buds arose from stele tissues and also from regenerated leaves (secondary organogenesis). Histological and SEM analyses showed a membranous-fibrillar structure, which was similar to the extracellular matrix (ECM), around non-morphogenic callus cells. The nuclear DNA content in leaves of plantlets regenerated *in vitro*, estimated using flow cytometry, was similar to donor plants (about 4.6 pg/2C). This is the first report concerning the micropropagation protocol for hybrid sorrel and the first DNA 2C-value estimation for this species.

1. Introduction

Rumex tianschanicus x Rumex patientia (hybrid sorrel, sorrel of Uteush), which is a prospective high energy productive crop, is a cross between English spinach (*Rumex patientia* L.) as the female line and Tien Shan sorrel (*Rumex tianschanicus* A. Los.) as the male line (Ust'ak and Ust'aková, 2004; Havlíčková and Suchý, 2010). This hybrid significantly exceeds both the original plants and many traditional feed crops in terms of the quality of feed production and the yields of above-ground biomass and seeds. Long-term trials have confirmed that the hybrid sorrel is one of the perennial energy crops with potential that is suitable for fuel biomass cultivation as a renewable source of energy in the European temperate-climate conditions (Ust'ak and Ust'aková, 2004). As such it can be used for biogas production and also for manufacturing pellets and briquettes (Myšková et al., 2011).

Renewable energy sources continue to play an important role in the energy policies of developed countries (Havlíčková and Suchý, 2010). A new biotechnological approach showed that energy plants also have a significant application for environment-friendly management, mainly in phytoremediation (the phytoextraction of heavy metals) technology, which was presented as a cleanup technology that belongs to the cost-effective and environment-friendly biotechnology (Masarovičová et al., 2009). According to Zhuang et al. (2005), *R. tianschanicus x R. patientia* has been proven to be heavy metal tolerant that has potential in the phytoremediation of soils that have been contaminated by multiple heavy metals.

The hybrid sorrel may be of interest not only as a fodder plant, but also as an important medicinal plant. The chemical composition of the hybrid, which was examined by Omarova et al. (1998), showed the presence of a high level of biologically active substances. e.g., carotenoids (provitamin A), ascorbic acid (vitamin C), linoleic and linolenic acids (vitamin F) and flavonoids (vitamin P).

Hybrid sorrel is an interesting object that has been studied in many physiological researches. Kosakivska et al. (2008) revealed that it is a plant with a high ecological plasticity, cold and winter hardiness and a tolerance to salt-stress and increased humidity.

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The results that were obtained by Kong et al. (2005) suggest that an appropriate concentration of exogenous selenium positively affects the antioxidant and osmoregulatory capacity and enhances the salt-tolerance in hybrid sorrel seedlings.

In order to improve the character of energy plants via genetic modification using biotechnological methods, we need to develop micropropagation techniques that deliver efficient long-term plant regeneration. This need has increased the importance of developing tissue culture methods to facilitate the large-scale production of high-quality planting material and for the improvement of species using genetic engineering technique (Kumar and Reddy, 2012). With an *in vitro* propagation method, unlimited plant material can consistently be obtained throughout the whole year. Additionally, genetically uniform plant material (less genetic diversity) can be produced easily. The protocol for *in vitro* propagation can also provide plant material for future physiological and biochemical studies (Turker et al., 2008).

The aims of the present experiments was to develop an efficient and rapid method of hybrid sorrel *in vitro* regeneration, to determine the pathway of morphogenesis by histological and SEM analyses and to compare the nuclear DNA content of donor plants and of regenerated plantlets using flow cytometry.

2. Materials and methods

2.1. Plant material and culture conditions

Seeds of *Rumex tianschanicus x Rumex patientia* cv. OK-2 that had been obtained from the Slovak University of Agriculture in Nitra were used. Fragments of the hypocotyls (ca. 5 mm), the cotyledons of 7- and 12-day-old seedlings and segments of petioles (ca. 5 mm) of 6-week-old plants growing in a phytotron chamber and in field conditions were used as explants.

The seeds were surface-sterilised in 70% ethanol for 5 min and then for 20 min in Ace commercial bleach (Procter & Gamble, Poland) that had been diluted with distilled water (3:1 v/v), followed by three rinses with sterile distilled water. Petioles that were derived from 6-week-old plants that were growing in a phytotron chamber (24 °C, 16/8 h photoperiod) were sterilised in 70% ethanol for 30 s and then for 5 min in Ace commercial bleach that had been diluted with distilled water (1:4 v/v), followed by three rinses with sterile distilled water.

MS culture media (Murashige and Skoog, 1962) supplied with the following types of auxin: 2.4-dichlorophenoxyacetic acid (2.4-D), indole-3-acetic acid (IAA), dicamba (Dic), indole-3-butyric acid (IBA), 1-naphthaleneacetic acid (NAA), picloram and/or cytokinins: thidiazuron (TDZ), kinetin, benzylaminopurine (BAP), and 2-isopentenyladenine (2iP) were tested. Additionally, media that had been supplemented with different types of sugars (sucrose, glucose and fructose) at different concentrations (1–3% and 6%) were used. 100 mg/l of ascorbic acid was added to some media in order to overcome the browning of explants as a result of the oxidation of polyphenols that were exuded from the cut surfaces of the explants (Turker et al., 2008). Fifty different culture media were tested in total (Table 1). Calli that were obtained on media 4, 6, 7, 9, 10, 20 and 28 (as induction media) were transferred onto regeneration media as described in Table 2.

The media were solidified with 0.8% agar (MP Biomedicals). The cultures were incubated at 26 ± 3 °C under a 16 h photoperiod (cool-white fluorescent tubes, 60–90 μmol photons m^{-2} s^{-1}). Five explants per Petri dish were inoculated and at least five replicates (Petri dishes) were used for each type of medium. In the case of the regeneration medium MS + 0.17 mg/l IAA + 2.2 mg/l BAP + 2% sucrose (coded as 40 in Table 1) 404 of 7-day-old and 401 of 12-day-old hypocotyls were used. The regeneration efficiency was evaluated by calculating the frequency of explants (%) that had formed shoot buds. Regenerated adventitious shoots were rooted on a ½ MS medium that had been supplemented with 2% sucrose and 0.5 mg/l IBA and acclimated in a phytotron chamber (24 °C, 16/8 h photoperiod) and then in field conditions.

2.2. Histological analysis

The material was prepared for embedding tissues in Technovit 7100 (2-hydroxyethyl-methacrylate) (Heraeus Kulzer) as

Table 1
Media tested in experiments based on MS (Murashige and Skoog, 1962), supplemented with different plant growth regulators and carbohydrates at different concentrations.

Mediumno.	Auxins (mg/l)	Cytokinins (mg/l)	Carbohydrates	Vitamin C (mg/l)
1	2,4-D (0.01)		Sucrose 1.5%	
2	2,4-D (2.0)	Kinetin (2.0)	Sucrose 1.5%	
3–10	2,4-D (0.25, 0.5, 0.75, 1.0, 2.0, 2.5, 20.0, 40.0)		Sucrose 3%	
11	2,4-D (0.5)		Sucrose 3%	(100.0)
12	2,4-D (1.0)		Sucrose 1%	
13, 14	2,4-D (1.0)		Glucose 1%, 3%	
15	2,4-D (5.0)	BAP (0.5)	Sucrose 3%	
16–18	2,4-D (0.5)	Dic (0.5, 1.0, 2.0)	Sucrose 3%	
19	2,4-D (0.5)	Dic (2.0)	Sucrose 3%	(100.0)
20	2,4-D (1.0)	Dic (0.5)	Sucrose 3%	
21	2,4-D (1.0)	Dic (0.5)	Sucrose 3%	(100.0)
22, 23	2,4-D (1.0)	Dic (1.0, 2.0)	Sucrose 3%	
24–26	2,4-D (2.0)	Dic (0.5, 1.0, 2.0)	Sucrose 3%	
27	2,4-D (5.0)	Kinetin (0.1)	Sucrose 3%	
28–30	Dic (0.5, 1.0, 2.0)		Sucrose 3%	
31	Dic (0.5)		Sucrose 3%	(100.0)
32	Dic (3.0)	TDZ (0.01)	Sucrose 3%	
33	Dic (0.3)	Kinetin (0.1)	Sucrose 3%	
34, 35	IAA (0.5)	Kinetin (1.0)	Glucose (1%, 3%)	
36, 37	IAA (0.5)	Kinetin (1.0)	Fructose (1%, 3%)	
38, 39	IAA (0.5)	Kinetin (1.0)	Sucrose (1%, 3%)	
40, 41	IAA (0.17)	BAP (2.2)	Sucrose (2%, 6%)	
42–45	Picloram (0.2, 4.0, 8.0, 12.0)		Sucrose 3%	
46, 47	2iP (2.0)	NAA (0.5,4.0)	Sucrose 3%	
48		TDZ (0.5)	Sucrose 3%	
49		BAP (1.0)	Sucrose 3%	
50	None	None	Sucrose 3%	

All media are based on basal MS, except media no. 1 and 2 (½ MS). Medium no. 40 was based on Ćulafić et al. (1987) report.