Plant Cell Tiss Organ Cult (2015) 123:193–203
DOI 10.1007/s11240-015-0826-z

ORIGINAL ARTICLE

Male adventitious roots of *Rumex thyrsiflorus* Fingerh. as a source of genetically stable micropropagated plantlets

Halina Ślesak[1] · Grzegorz Góralski[1] · Dagmara Kwolek[1] · Katarzyna Dziedzic[1] · Aleksandra Grabowska-Joachimiak[2]

Received: 10 March 2015 / Accepted: 22 July 2015 / Published online: 30 July 2015

Abstract *Rumex thyrsiflorus* Fingerh. is one of the few dioecious plant species that have sex chromosomes. The chromosome constitution of females is $2n = 12A + XX$ and $2n = 12A + XY_1Y_2$ of males. It is a medicinally important plant species and has also been the object of studies on the structure and function of sex chromosomes and sex ratio. An efficient plant regeneration protocol was developed from karyologically stable male roots that had been derived from a long-term liquid culture. The root segments were grown on MS medium supplemented with the following plant growth regulators: 2.4-D, NAA, kinetin, BAP and TDZ. The highest frequency (81.73 %) of adventitious shoot formation (16.27 shoots/explant) was obtained on MS + 0.5 mg/l TDZ. Regenerated shoots were successfully rooted on ½ MS + 2 % sucrose + 0.5 mg/l IBA and acclimated to in vivo conditions. Histological analysis revealed indirect (via callus) adventitious shoot formation. The cells of the morphogenetic callus were surrounded by a fibrillar structure that was similar to the extracellular matrix. Molecular analysis based on genetic sex markers confirmed that all of the root explants were male. The genetic stability of the regenerated plantlets was confirmed using random amplified polymorphic DNA analysis. This is the first report concerning the micropropagation protocol for *R. thyrsiflorus* Fingerh. from male roots derived from a long-term liquid culture, which offers a unique opportunity to obtain true-to-type plants of the same sex.

Keywords In vitro root culture · Micropropagation · Organogenesis · Histological analysis · Molecular markers · Genetic stability

Introduction

Rumex thyrsiflorus Fingerh. (pyramidal sorrel, thyrse sorrel) is one of the few dioecious plant species that have sex chromosomes. It is closely related to *R. acetosa*, which is a model species in plant sex chromosome studies. Both species are difficult to distinguish, form mixed populations and can interbreed with each other (Grabowska-Joachimiak et al. 2012). The chromosome constitution of *R. thyrsiflorus* females is $2n = 12A + XX$ and of males is $2n = 12A + XY_1Y_2$ (Żuk 1963). The species is an attractive subject for studies on the structure and function of sex chromosomes and biased sex ratios in populations and seeds (Rychlewski and Zarzycki 1986; Kwolek and Joachimiak 2011; Grabowska-Joachimiak et al. 2012).

According to Litvinenko and Muzychkina (2008), *R. thyrsiflorus* was introduced to medicine in 2006 because of phytopreparations that are known to exhibit hemostatic, astringent, anti-inflammatory, antiscorbutic, antiseptic, diuretic, analgesic, antitumor and antihelminthic activity. The varied biological activity of sorrel is due to the presence of various groups of biologically active substances, in particular, flavonoids, tanning agents, phenolic acids, anthraquinones, polyunsaturated fatty acids etc.

Recently, Lajter et al. (2013) reported that noteworthy antiproliferative activities for cancer cells were recorded

✉ Halina Ślesak
halina.slesak@uj.edu.pl

[1] Department of Plant Cytology and Embryology, Institute of Botany, Jagiellonian University, Gronostajowa St. 9, 30-387 Cracow, Poland

[2] Department of Plant Breeding and Seed Science, University of Agriculture in Cracow, Łobzowska St. 24, 31-140 Cracow, Poland

for several *Rumex* species. It was found that extracts of *R. thyrsiflorus* at 10 or 30 mg/ml demonstrated substantial cell growth inhibitory activity (at least a 50 % inhibition of cell proliferation) against one or more cell lines and it proved to be the most active species and demonstrated a strong anticancer profile. In view of its pronounced antiproliferative activities, *R. thyrsiflorus* is worthy of further investigations, including bioassay-guided isolation and the identification of the active substances and has revealed itself to be a promising candidate for further activity-guided fractionation in the search for new active antitumour compounds (Lajter et al. 2013).

In recent years, tissue culture techniques have been intensively used for the propagation of medicinally important plant species, which are important sources of compounds for the pharmaceutical industries (Parveen and Shahzad 2011). It is important to note that the roots are the principal plant material from which drugs are prepared (Sudha and Seeni 2001) and that root culture is an alternative method of both clonal propagation and germplasm conservation (Bernabé-Antonio et al. 2010). Additionally, root explants are advantageous over other explants in terms of their easy manipulation and higher degree of regeneration potential (Franklin et al. 2004). In the case of plants with sex chromosomes, developing a method of in vitro micropropagation from this type of explants could offer a unique opportunity to obtain true-to-type plants of the same sex, which is essential in cytogenetic research.

The long-term, karyologically stable, liquid cultures of adventitious roots of sorrel were obtained by Mosiołek et al. (2005). The aim of our preliminary molecular analysis based on species-specific DNA markers, that were developed by Grabowska-Joachimiak et al. (2012), was to verify the sex of these adventitious roots and to confirm, that they were obtained from *R. thyrsiflorus*. It is known that *R. thyrsiflorus* is morphologically similar to *R. acetosa* (the species are difficult to distinguish) and therefore it is treated by some authors as its subspecies [*R. acetosa* subsp. *thyrsiflorus* (Fingerh.) Hayek] (Kwolek and Joachimiak 2011).

The aim of the subsequent experiments was to develop a simple and efficient in vitro micropropagation method for *R. thyrsiflorus* from male root explants, to determine the pathway of morphogenesis using a histological analysis and to evaluate the genetic stability of the micropropagated plantlets using molecular markers.

Materials and methods

Plant material and culture conditions

The long-term culture of *R. thyrsiflorus* adventitious roots (line RCY) was obtained as described by Mosiołek et al. (2005). They were cultured on a gyratory shaker (100 rpm) in 250 ml Erlenmeyer flasks containing 35 ml of MS (Murashige and Skoog 1962) liquid medium with ½ strength macronutrients without plant growth regulators. Different flasks were described as RCY1, RCY2, RCY3 and RCY4. The explants, 5 mm long adventitious roots, obtained from a long-term liquid cultures (8 years old), were inoculated on a solid MS medium that had been supplemented with different concentrations of following plant growth regulators: kinetin, 1-naphthaleneacetic acid (NAA), benzylaminopurine (BAP), thidiazuron (TDZ), 2.4-dichlorophenoxyacetic acid (2.4-D) and different concentrations of sucrose (3 or 12 %), as is presented in Table 1. 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 $m^{-2}s^{-1}$). Three explants per Petri dish were inoculated and twenty replicates (Petri dishes) were used for each type of medium, except for medium no. 6 (coded as is shown in Table 1) (104 replicates). The regeneration efficiency was evaluated by calculating the frequency of explants (%) that had formed shoot buds. Regenerated adventitious shoots were rooted on: (1) MS without plant growth regulators, (2) MS supplemented with 0.5 mg/l indole-3-butyric acid (IBA) or 0.5 mg/l indole-3-acetic acid (IAA), (3) ½ MS supplemented with 2 % sucrose and 0.5 mg/l IBA or 0.5 mg/l IAA or (4) by rinsing in IBA solutions (20 mg/l) for 24 h followed by inoculation on MS medium without plant growth regulators. Rooted plantlets were acclimated in a phytotron chamber (24 °C, 16/8 h photoperiod) and then in field conditions.

Histological analysis

Histological analysis was performed on explants that had been cultured on MS + 0.5 mg/l TDZ, which were collected after 3–6 weeks of the culture. The material was prepared for embedding tissues in Technovit 7100 (2-hydroxyethyl-methacrylate) (Heraeus Kulzer, Germany). The explants were fixed in 5 % buffered (0.1 M phosphate buffer, pH 7.2) glutaraldehyde at room temperature for 24 h, washed four times in the same phosphate buffer (PBS) followed by dehydration in a graded ethanol series (10, 30, 50, 70, 96 %) for 15 min at each concentration and kept overnight in absolute ethanol. Later, the samples were infiltrated in a mixture of absolute ethanol and Technovit (1 h at each proportion: 3:1, 1:1, 1:3; v/v) and stored for 12 h in pure Technovit. The resin was polymerised by adding a hardener. The material was sectioned to 5 µm using a rotary microtome (Microm, Adamas Instrumenten), stained with 0.1 % toluidine blue O (TBO) and mounted in Entellan synthetic resin (Merck, Germany).

Table 1 The media that were tested in the experiments that were based on MS (Murashige and Skoog 1962), and supplemented with different plant growth regulators at different concentrations and the frequency of the morphogenetic response of explants (number of responsive explants/explants used)

Medium no.	Auxins (mg/l)	Cytokinins (mg/l)	Sucrose (%)	No. of explants used/no. of responsive explants
1	NAA (1.0)		3.0	60/0
2	2,4-D (1.0)		3.0	60/0
3		Kinetin (1.0)	3.0	60/0
4		BAP (0.6)	3.0	60/1
5		TDZ (0.1)	3.0	60/11
6		TDZ (0.5)	3.0	312/255
7		TDZ (2.0)	3.0	60/9
8	NAA (1.0)	BAP (0.3)	3.0	60/0
9	NAA (1.0)	Kinetin (2.0)	3.0	60/0
10			3.0	60/0
11			12.0	60/0

Microscopic sections were photographed using a Nikon DS-Fi2 with NIS-Elements D 4.00.00 4.0 software.

Molecular analysis

Genomic DNA was extracted from plant material (roots from the liquid culture, the callus tissue and the leaves of regenerated plantlets) using the hexadecyltrimethylammonium bromide (CTAB) method (Gawal and Jarret 1991) with modifications (Kwolek and Joachimiak 2011).

To confirm that the roots that were used as explants were *R. thyrsiflorus* roots (the species *acetosa* and *thyrsiflorus* are difficult to distinguish) and to confirm the sex of the cultured roots of *R. thyrsiflorus*, PCR-based methods, which involved the DNA markers that are located on Y chromosomes were used. The following primers, which were developed by Korpelainen (2002), RAY-F (5′-ACTCGAATGTAAGCATTTGGTCCTA-3′) and RAY-R (5′-ACTACACGATTGTCCATAAAGTGGA-3′) were used to amplify the male-specific RAYSI sequence that was present on the Y chromosomes of *R. acetosa* and its close relatives (Navajas-Peréz et al. 2006). The polymerase chain reaction (PCR) mixture (15 µl) contained a 1×Taq Polymerase buffer (Thermo Scientific), 5 mM $MgCl_2$, 0.25 mM dNTPs, 0.25 mM of each primer, 1.125 U Taq DNA Polymerase (Thermo Scientific) and approximately 15 ng of the template DNA.

Amplifications were performed using the following programme: an initial denaturation step at 94 °C for 4 min., 30 cycles consisting of a denaturation step at 94 °C for 1 min., a primer annealing step at 60 °C for 45 s and a primer extending step at 72 °C for 1 min. 30 s and a final extending step at 72 °C for 8 min. The PCR products were separated in 1 % agarose gel using Simply Safe (EURx). Additionally, UGR08-F (CCAATTGGTCTCAACTAGAACA) and UGR08-R (TGTTATAGGTTTTGGACTGCCA), which are primers that are specific for the male-specific repetitive sequence RAYSII in *R. acetosa* L. (Mariotti et al. 2009), were used. In this case, PCR amplification and visualisation were conducted as they are for the RAY-F and RAY-R primers. To verify the template DNA quality, amplification with primers R730-A (5′-CTCGGACCAATTATCTCAT-3′) and R730-B (5′-CATTATTTGGGAGCCGAT-3′) (Navajas-Peréz et al. 2005) was carried out. These primers amplify the repetitive RAE730 sequence that is located on the *Rumex* autosomes. The reaction mixture and programme were the same as described above except for the temperature of the primer annealing step (55 °C). The amplification reaction was carried out in a T100 Thermal Cycler (BioRad).

Genetic uniformity between the adventitious roots from the liquid culture, the callus tissue and the plantlets that had been regenerated in vitro from the roots was assessed using PCR-based random amplified polymorphic DNA (RAPD) analysis. The RAPD assay was performed using ten random decanucleotide primers (Table 2).

The RAPD was performed in a 10 µl reaction mixture containing 10 ng of the template DNA, a 1×Taq Polymerase buffer (Thermo Scientific), 2 mM $MgCl_2$, 0.25 mM dNTPs, 1 mM random primer and 0.5 U Taq DNA Polymerase (Thermo Scientific). The amplification reaction was carried out in a TC-Plus thermal cycler (TECHNE).

The PCR programme consisted of an initial denaturation at 94 °C for 1 min followed by 39 cycles of denaturation at 93 °C for 30 s, primer annealing at 42 °C for 30 s, extension at 68 °C for 2 min. 30 s and a final extension at 68 °C for 2 min. 30 s.

Amplification with each primer was repeated twice in order to confirm the reproducibility of the results and only

Table 2 RAPD primer sequences and amplification results

Primer	Sequence (5′–3′)	Size range (bp)	No. of amplified bands	No. of polymorphic bands
RAPD1	GCAAGTAGCT	420–1800	10	0
RAPD3	CAGTGTGTGG	320–2200	14	0
RAPD4	GTGTCAGGCA	380–2100	13	0
RAPD7	GATAACCGCA	450–1750	8	1
RAPD8	ATCCGCGTTC	600–2500	11	1
RAPD10	TGACGATGCA	450–2300	13	1
RAPD13	ATGTCCGCAC	380–2000	9	1
RAPD14	GTGTGGATGG	550–1750	7	0
OPA-04	AATCGGGCTG	450–2900	13	2
OPB-07	GGTGACGCAG	450–2000	12	1
Total		320–2900	110	7

The sequences of the primers RAPD1–RAPD14 were based on Sakamoto et al. (1995), OPA-04 and OPB-07 derived from Operon (http://www.eurofinsdna.com/home.html)

clear bands that were amplified in both duplicates were analysed. The amplified samples were analysed using electrophoresis in 1 % agarose gel with a 1xTBE buffer and stained with ethidium bromide.

The GeneRuler 100 bp PLUS DNA Ladder (Thermo Scientific) was used as the molecular standard.

Data analysis

The statistical tests and graph for the efficiency of the micropropagation were done using the R environment for statistical computing version 3.1.2 (R Development Core Team 2014). Any differences between the results of the cultures were compared using the test of equal or given proportions (prop. test from stats library). The same test was used for estimating the 95 % confidence intervals that are shown in Fig. 2.

The RAPD fingerprints that were generated were individually scored. The bands were transformed into a binary character matrix, "1" for presence and "0" for absence of band at a particular position.

Faint and ambiguous bands were not included in the statistical analysis. The RAPD profiles were compared for 15 samples of adventitious roots from the liquid culture and for 19 plantlets that had been regenerated in vitro from roots. Additionally, we compared the samples of RCY1 and RCY2 roots, selected callus tissue that had been induced on them and plantlets that had been regenerated from these calli. The percentage of polymorphism was calculated as the ratio of the number of polymorphic bands to the total number of bands.

Results

Plant regeneration from the liquid root culture

Callus induction was observed on the explants 2 weeks after the inoculation of the roots obtained from a long-term liquid cultures (Fig. 1a) on solid medium. Callus tissue was visible on roots cultured on the MS medium supplemented with following plant growth regulators: 1 mg/l 2.4-D, 0.6 mg/l BAP and TDZ (media 2, 4–7 coded as in Table 1). There were observed morphological differences in callus tissue depending on the media. On the medium with 1 mg/l 2.4-D, the callus tissue was white and well hydrated in contrast to the medium supplemented with 0.6 mg/l BAP, which induced a green and compact callus. The MS medium supplemented with TDZ produced a callus mass with optimal growth. The induction of the callus started at the cut ends of the roots, and later covered the entire surface of the explants (Fig. 1b). The highest intensity and efficiency of callogenesis (100 % of explants forming callus) was observed on the MS supplemented with 0.5 mg/l TDZ. Only media supplied with different TDZ concentration (0.1, 0.5 and 2.0 mg/l; media 5–7 coded as in Table 1) resulted in a significant morphogenetic response of the cultured explants (Table 1; Fig. 2). The most efficient proved to be the MS medium supplemented with 0.5 mg/l TDZ, where 81.73 % of explants showed adventitious shoots induction (16.27 shoots/explant), after 6 weeks from the beginning of the culture (Figs. 1c, d, 2). Two-week-old adventitious shoots were isolated and inoculated on rooting media (Fig. 1e), among which the best results in roots induction were obtained on ½ MS + 2 % sucrose + 0.5 mg/l IBA (almost 100 % efficiency). In this case, rhizogenesis was noted after ca 2 weeks (Fig. 1f). Rooted plantlets were successfully acclimated to in vivo (Fig. 1g) and subsequently to field conditions (Fig. 1h).

Histological analysis

A longitudinal section of roots that had been cultured on MS + 0.5 mg/l TDZ for 3 weeks revealed cell divisions and callus differentiation in the cortex layer (Fig. 3a). The callus tissue was heterogeneous and was composed of cells

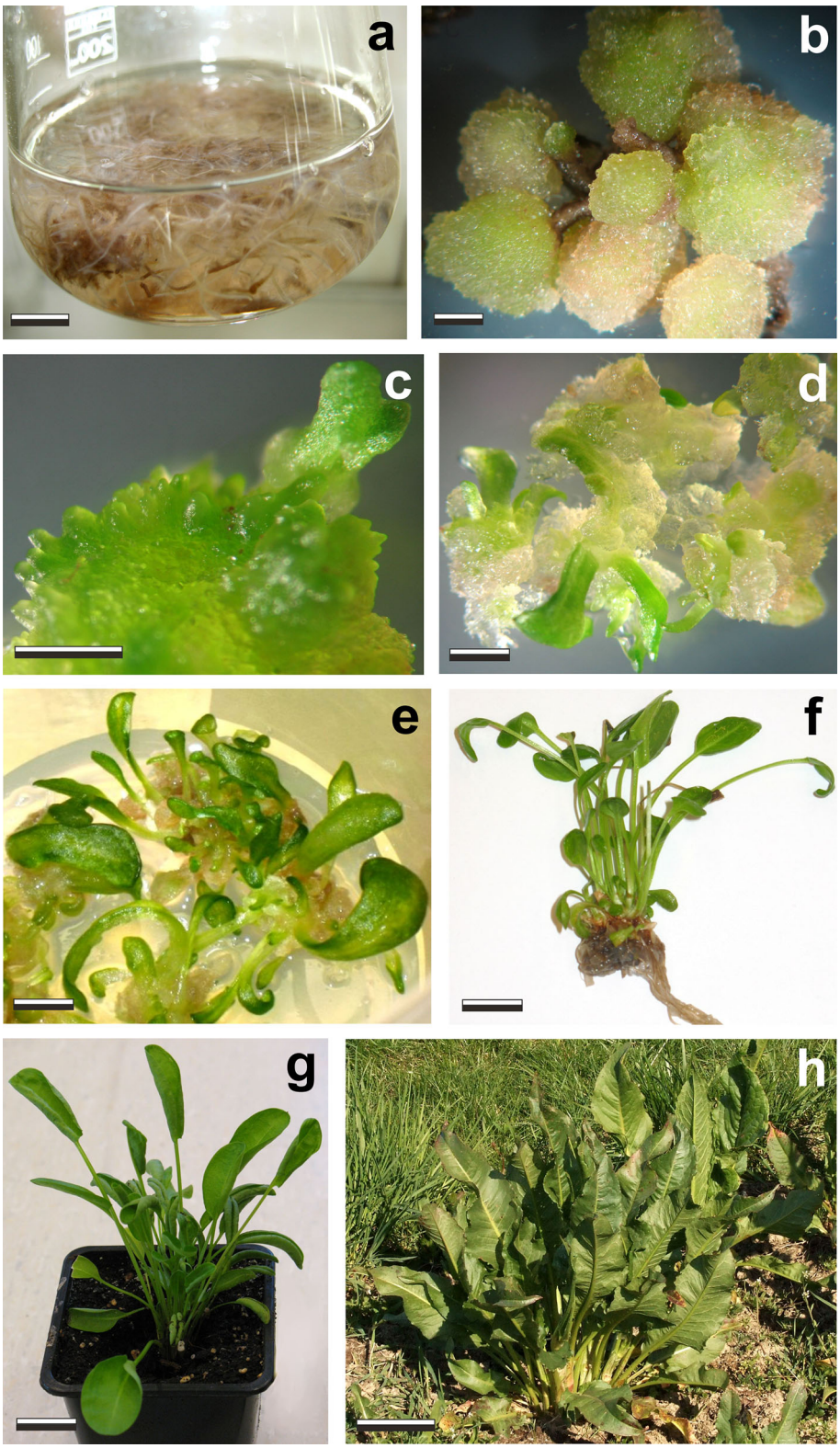

Fig. 1 Callus induction and plant regeneration on MS + 0.5 mg/l TDZ. Adventitious roots cultured on MS liquid medium with ½ strength macronutrients without plant growth regulators (**a**); callus induction on roots 3 weeks after the beginning of the culture (**b**); induction of adventitious shoots six (**c**) and seven (**d**) weeks after the beginning of the culture; isolated 2-week-old shoots that had been inoculated on rooting medium ½ MS + 2 % sucrose + 0.5 mg/l IBA (**e**); rooted plantlet (**f**) acclimated to in vivo (**g**) and field (**h**) conditions. *Bars* 1 mm (**b**, **c**), 3 mm (**d**), 10 mm (**a**, **e**), 20 mm (**f**, **g**), 50 mm (**h**)

that varied in shape, size and the degree of vacuolisation (Fig. 3b–d). Small, isodiametric, dividing cells with dense cytoplasm formed meristematic centres (Fig. 3b), which were visible deep inside the callus (Fig. 3c) and in the surface layers of the callus tissue (Fig. 3d). Large, highly vacuolated callus cells were loosely attached each other

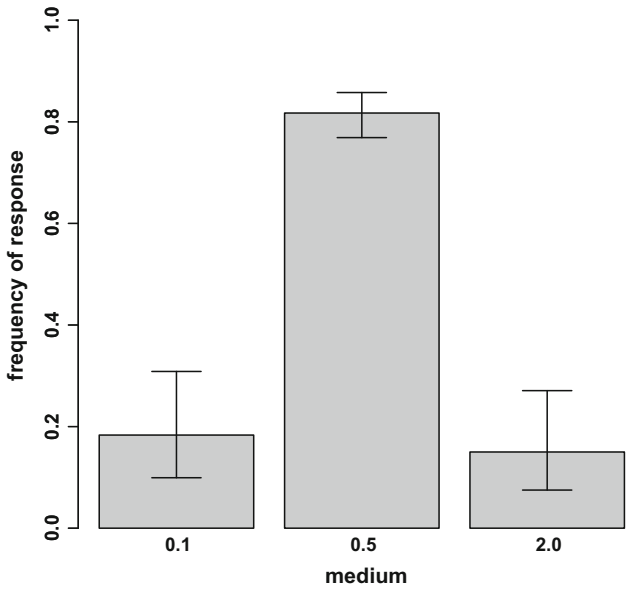

Fig. 2 Frequency of the morphogenetic response of the adventitious root culture (percentage of explants with shoots) on media with different TDZ concentrations (media 5–7 coded as in Table 1). *Error bars* indicate 95 % CI

(Fig. 3e, f). Histological analyses of the morphogenetic callus showed a fibrillar structure in the intercellular spaces between the callus cells, which was similar to the extracellular matrix (ECM; Fig. 3e, f). Additionally, starch grains were visible inside the morphogenetic callus cells (Fig. 3g). It was well demonstrated that the meristematic zones enlarged in size because of the fast meristematic activity that leads to the formation of nodular structures. Further development led to the differentiation of the leaf primordia and the shoot apical meristem. Cross sections of the explants that had been cultured 6 weeks revealed typical looking regenerated plantlets with a visible shoot apex and leaf primordia (Fig. 3h).

Molecular analysis

The amplification of the male-specific repetitive sequence RAYSI on the Y chromosomes using RAY-F and RAY-R primers showed the presence of approximately a 930 bp PCR product of this reaction was obtained for all of the adventitious roots that were used as explants that were analysed (Fig. 4a). The amplification of the sequence RAYSII using the primers UGR08-F and UGR08-R resulted in obtaining a product of the same size (around 700 bp) that occurred in all of the samples (Fig. 4b), which confirmed that the roots from the liquid culture that had been used as explants were male. They also had an additional amplification product of around 600 bp, which is characteristic for *R. thyrsiflorus*. Amplification with primers R730-A and R730-B resulted in obtaining PCR products for all of the samples that were tested, which indicated that the DNA templates that had been used for sex determination were of good quality (Fig. 4c).

The ten RAPD primers that had been selected gave rise to a total of 110 distinct bands that ranged from 320 to 2900 bp in size (Table 2). The number of bands scored in each primer varied from 7 in the RAPD14 primer to 14 in the RAPD3 primer with an average of 11 bands per primer. A high level of similarity was revealed by the RAPD banding pattern in the callus and regenerated plantlets and most of the primers showed DNA profiles that were identical to those of the adventitious roots that had been used as explants.

Of the 110 amplification products, only 7 were polymorphic (6.36 % bands), while the rest were monomorphic. The representative profiles of the male adventitious roots from the liquid culture and the plantlets that had been regenerated in vitro with primer RAPD14 are presented in Figs. 5 and 6. The DNA amplification profiles of the selected callus tissue that had been induced on adventitious roots and plantlets that had been regenerated from callus that had been obtained with RAPD10 are presented in Fig. 7.

Discussion

During these experiments, a reproducible micropropagation protocol was developed for *R. thyrsiflorus* by using high frequency plant regeneration from root-derived callus.

The origin and nature of the plant material play an important role in the success of micropropagation. Among the different organs, roots have been investigated as a source of explants for shoot induction in very few species such as *Populus tremula* (Vinocur et al. 2000), *Clitoria ternatea* (Shahzad et al. 2007), *Casia angustifolia* (Parveen and Shahzad 2011) or *Centaurea ultreiae* (Mallón et al. 2011). The use of roots in large-scale bioreactor cultures has been reported in *Eleutherococcus koreanum* (Park et al. 2005).

Our experiments indicated that the adventitious roots of *R. thyrsiflorus* obtained from a long-term liquid cultures (8 years old), have the potential to form adventitious shoots. The best morphogenetic response we obtained was on the MS medium supplemented with TDZ. The highest efficiency of adventitious shoots induction and the highest average number of shoots per explant was obtained on the MS supplied with 0.5 mg/l TDZ. Among the other culture media that were tested only the medium with BAP resulted in adventitious shoot induction, although only on one explant. Similar results were obtained by Parveen and Shahzad (2011) in a *Cassia angustifolia* root culture in which TDZ, among investigated cytokinins, was the most effective in the induction of an organogenic callus for

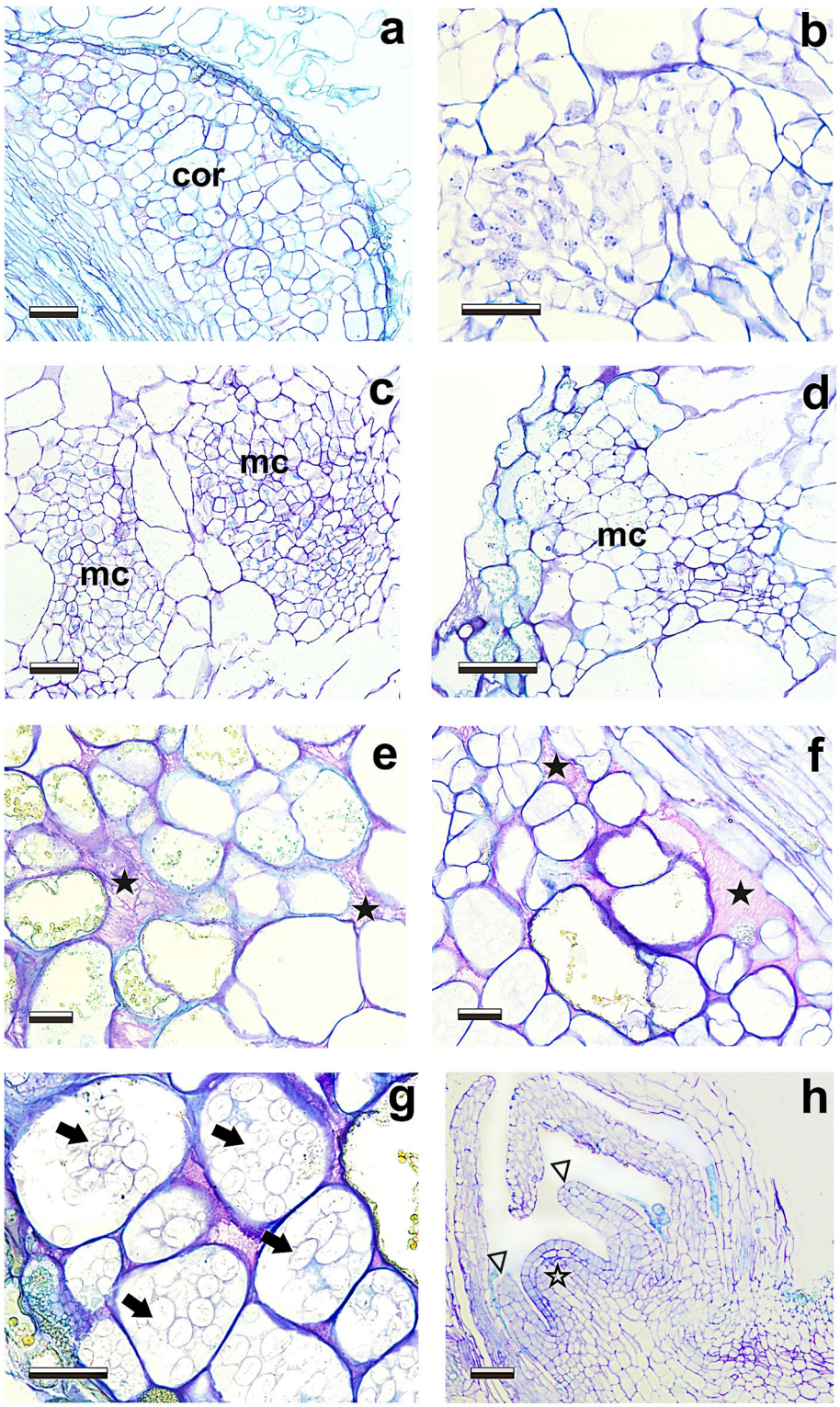

Fig. 3 Histological sections of explants that had been cultured on a regeneration medium MS + 0.5 mg/l TDZ. Longitudinal section of a root after 3 weeks of culture (**a**), visible cortex (*cor*), note cell divisions and callus differentiation. Cross section of roots after 4 weeks of culture; visible meristematic centres (*mc*) with dividing cells (**b**) located deep inside the callus (**c**) and on the callus surface (**d**). Morphogenetic callus cells surrounded by an ECM-like fibrillar structure (*black asterisks*) (**e**, **f**) and cells filled by starch grains (**g**) (*black arrows*) (4 weeks of culture). Plantlet regenerated on the surface of the friable callus (**h**), visible shoot apex (*white asterisk*) and leaf primordia (*white arrowheads*) (6 weeks of culture). All sections (**a**–**h**) stained with toluidine blue. *Bars* 100 µm (**e**–**h**), 200 µm (**a**–**d**)

multiple shoot regeneration. Similarly, Ma et al. (2011) indicated that TDZ has a stronger effect on callus induction and shoot organogenesis than BAP in *Ochna integerrima*. TDZ can act as a substitute for both the auxin and cytokinin requirements for in vitro morphogenesis (organogenesis and somatic embryogenesis) in several species (Murthy et al. 1998). Its mode of action may be attributed to its ability to induce cytokinin accumulation and/or to enhance the accumulation and translocation of auxin (Murch and Saxena 2001).

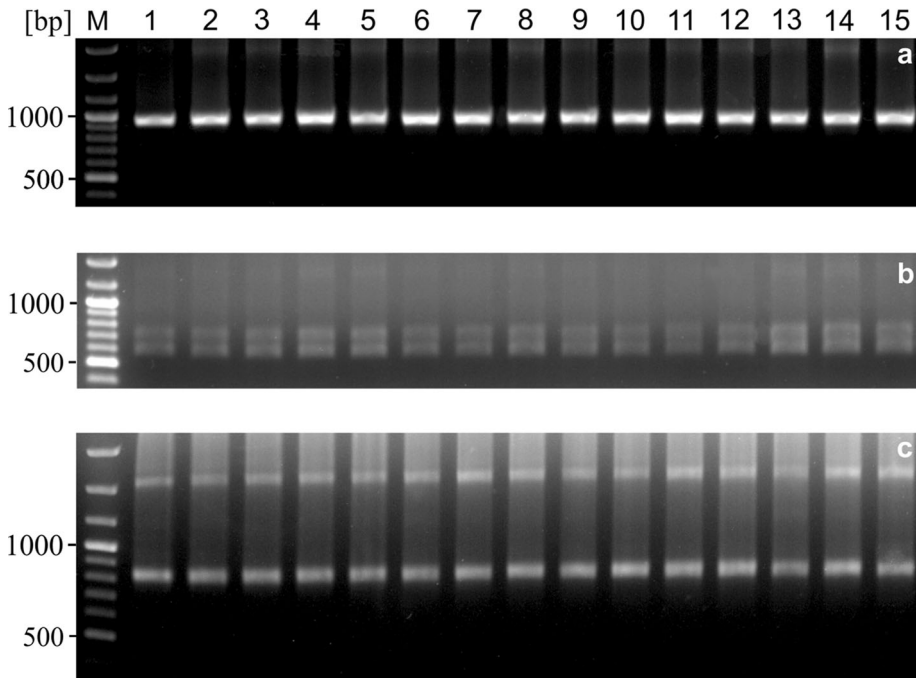

Fig. 4 PCR products in *Rumex thyrsiflorus* root explants (1–15): RAY-F and RAY-R primers (**a**), UGR08-F and UGR08-R primers (**b**), R730-A and R730-B primers (**c**), M-100 bp molecular weight marker

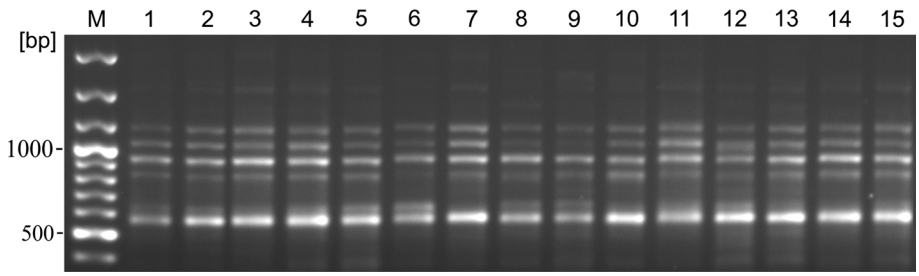

Fig. 5 DNA amplification profiles of adventitious roots from a liquid culture (1–15) obtained using the RAPD14 primer: 1–4: RCY1, 5–8: RCY2, 9–11: RCY3, 12–15: RCY4, M-100 bp molecular weight marker

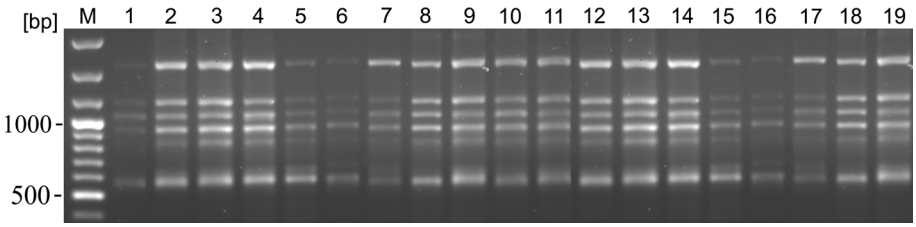

Fig. 6 DNA amplification profiles of plantlets that had been regenerated in vitro (1–19) that were obtained using a RAPD14 primer: 1–8: plantlets that had been regenerated on RCY1 roots, 9–14: on RCY2 roots, 15–18: on RCY3 roots, 19: on RCY4 roots, M-100 bp molecular weight marker

Carbohydrates are essential biomolecules that are necessary for the growth and development of plants in vitro (Bogunia and Przywara 2000). According to Jach and Przywara (2000), different patterns of morphogenesis are attributable to the type of carbohydrate and its concentration. A high sucrose concentration (12 %) in a culture medium resulted in the induction of somatic embryogenesis on immature zygotic sunflower embryos, in contrast to a low sucrose concentration, which induced an organogenetic response. Our experiments concerning *R. thyrsiflorus* organogenesis revealed that media that had been supplemented with 12 % of sucrose failed to have a morphogenetic response.

The pattern of regeneration in *R. thyrsiflorus* was also studied through histological sections, which confirmed that morphogenesis proceeded by the indirect (via callus) formation of adventitious shoots. The cells of the morphogenetic callus were surrounded by a fibrillar structure

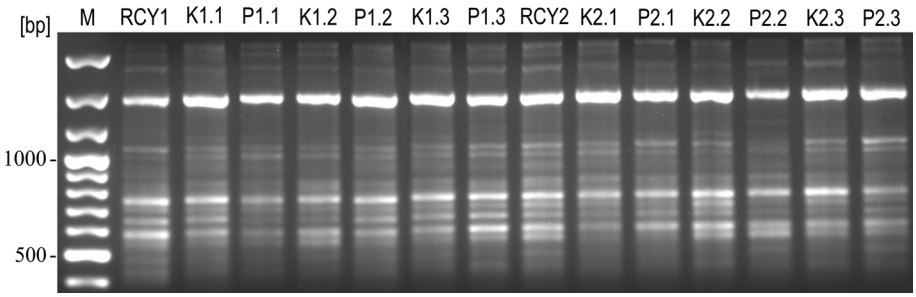

Fig. 7 DNA amplification profiles of the roots (RCY1, RCY2), callus and regenerated plantlets that were obtained using a RAPD10 primer: K1.1–P1.1: callus 1 that had been induced on RCY1 and a plantlet that had been regenerated from callus 1, K1.2–P1.2: callus 2 that had been induced on RCY1 and a plantlet that had been regenerated from callus 2, K1.3–P1.3: callus 3 that had been induced on RCY1 and a plantlet that had been regenerated from callus 3, K2.1–P2.1: callus 1 that had been induced on RCY2 and a plantlet that had been regenerated from callus 1, K2.2–P2.2: callus 2 that had been induced on RCY2 and a plantlet that had been regenerated from callus 2, K2.3–P2.3: callus 3 that had been induced on RCY2 and a plantlet that had been regenerated from callus 3, M-100 bp molecular weight marker

similar to the ECM. ECM has been reported in plant tissue cultures of different species and may be formed on the surface of tissue that is cultured under in vitro conditions as a stress response or protective layer against external factors regardless of its morphogenetic competence (Pilarska et al. 2014; Ślesak et al. 2014). ECM can also serve as a structural marker of somatic embryogenesis (Namasivayam et al. 2006) or organogenesis (Popielarska-Konieczna et al. 2008, 2010), which was confirmed in our experiments on *R. thyrsiflorus*, in which we observed a fibrillar structure that surrounded morphogenetic callus cells.

According to Grabowska-Joachimiak et al. (2012), the results of the amplification of the sequence RAYSII using the UGR08-F and UGR08-R primers differed between *R. acetosa* and *R. thyrsiflorus* by the presence of a single product (~700 bp) in *R. acetosa* and by the presence of two products (~600 and ~700 bp) in *R. thyrsiflorus*. During our experiments, the amplification of the sequence RAYSII with these primers resulted in obtaining a ~700 bp product in all of the roots that were analysed, which indicates that all of the explants were male. They also showed an additional DNA fragment with a size of around 600 bp, which is characteristic for *R. thyrsiflorus*.

During micropropagation experiments it is, therefore, essential to establish the genetic uniformity of cultured plantlets in order to ensure the quality of plantlets for their commercial value and the application of biotechnology for micropropagation of true-to-type clones, for example (Eshraghi et al. 2005). Molecular DNA techniques are at the present, most common, cost effective method and valuable tools for establishing the genetic uniformity of micropropagated plantlets (Fatima et al. 2012). During our experiments, we used RAPD for the analysis of the genetic uniformity of the regenerated plantlets, as this is technically simple, quick to perform, requires very little plant material and yields true genetic markers and above all, has been used, and has proven to be an efficient tool for assessing genetic stability in the tissue culture process (Qin et al. 2006; Kengkarj et al. 2008). Although other molecular markers such as amplified fragment length polymorphism (AFLP) are more reproducible and require small amounts of DNA, they are more sophisticated, require special equipment and are more expensive than RAPDs (Agarwal et al. 2008).

The roots from a long-term liquid cultures that were used as explants in our experiments appeared to be karyologically stable. According to Mosiołek et al. (2005), no mitotic disturbances are observed in them and all of the cells that were analysed invariably had the 15 chromosomes (12 autosomes + XY_1Y_2) of a standard morphology. The positive results that were obtained using UGR08 (UGR08-F and UGR08-R) and RAY (RAY-F and RAY-R) primers showed that all of the roots that were analysed maintained their Y chromosomes despite 8 years of in vitro culture. All of the plants that were regenerated from these roots and acclimated to field conditions were typical males that produced only staminate flowers. This also suggests a lack of major changes in the rest of the chromosome complement, because the sex in *Rumex acetosa* and its close relatives depends on the X/autosome balance (Parker and Clark 1991).

During our experiments a high level of genetic similarity was revealed by RAPD banding pattern in the callus and the regenerated plantlets and most of the primers showed DNA profiles that were identical to the adventitious roots that were used as explants. Of the 110 amplification products, only seven were polymorphic (6.36 %), while the rest were monomorphic. It should be emphasised that the regeneration system described here appeared to be genetically stable in spite of callus tissue formation. It cannot be excluded that only callus cells without any mutation can have a morphogenetic potential and are favored during organogenesis. This is an important and interesting observation because generally regeneration via callus is regarded

as undesirable because of the variability of this tissue both on the chromosomal and DNA levels (Bayliss 1980; Gernand et al. 2007; Neelakandan and Wang 2012; Mizia et al. 2014). It is possible that the small genetic alterations that were observed in the regenerated plants can be explained by the kind of explants that were used in the culture. The roots were obtained from a long-term culture and it is possible that over many years in in vitro conditions some kind of selection occurred, which resulted in the survival of the most genetically stable clones. That explanation needs future studies and if confirmed could open up a new way for obtaining genetically stable material for biotechnological purposes.

Recently, RAPD analysis was used as an efficient tool to evaluate the clonal fidelity of micropropagated plants in many systems and indicated that the pattern of monomeric bands that are observed in *Ajuga bracteosa* (Kaul et al. 2013), *Spilanthes calva* (Razaq et al. 2013) and *Rhinacanthus nasutus* (Cheruvathur and Thomas 2014) are in agreement with our observations.

The plantlets regenerated from roots during our experiments exhibited normal morphological characters and no detectable variation was recorded in their morphology.

The results that were obtained suggest that an adventitious root culture of *R. thyrsiflorus* may be a source of genetically stable true-to-type regenerants, which seems to be very important in genetic studies on sex chromosomes and sex determination in this species. Some preliminary studies on genetic stability of *R. thyrsiflorus* regenerants derived from hypocotyls revealed a high degree of polymorphism. Among 124 amplification products, 54 were polymorphic (43.55 %) (Dziedzic, unpubl.). It should be emphasised that the root explant harbours the least amount of chimeric tissues, as the regenerated plants are proven to be genetically identical (Sharma et al. 1993).

In conclusion, the present study describes a novel, simple, very efficient and reliable protocol for indirect shoot organogenesis and multiplication of genetically stable *R. thyrsiflorus* plantlets from root explants. To the best of our knowledge, this is the first report on a tissue culture study in *R. thyrsiflorus* that offers a unique opportunity to obtain a large number of true-to-type plants of the same sex.

Acknowledgments The work was supported by statutory research funds of the Department of Plant Cytology and Embryology, Faculty of Biology and Earth Sciences, Jagiellonian University in Krakow.

References

Agarwal M, Shrivastava N, Padh H (2008) Advances in molecular marker techniques and their applications in plant science. Plant Cell Rep 27:617–631

Bayliss MW (1980) Chromosomal variation in plant tissues culture. Int Rev Cytol Suppl 11a:113–144

Bernabé-Antonio A, Estrada-Zúñiga ME, Buendía-González L, Reyes-Chilpa R, Chávez-Ávila VM, Cruz-Sosa F (2010) Production of anti-HIV-1 calanolides in a callus culture of *Calophyllum brasiliense* (Cambes). Plant Cell, Tissue Organ Cult 103:33–40

Bogunia H, Przywara L (2000) Effect of carbohydrates on callus induction and regeneration ability in *Brassica napus* L. Acta Biol Crac Ser Bot 42:79–86

Cheruvathur MK, Thomas TD (2014) Shoot organogenesis from root-derived callus of *Rhinacanthus nasutus* (L.) Kurz. and assessment of clonal fidelity of micropropagted plants using RAPD analysis. Appl Biochem Biotechnol 172:1172–1182

Eshraghi P, Zarghami R, Ofoghi H (2005) Genetic stability of micropropagated plantlets in date palm. J Sci Iran 16:311–315

Fatima N, Ahmad N, Anis M (2012) Rapid in vitro multiplication and genetic fidelity analysis in *Cuphea procumbens* Orteg., a plant rich in medium-chain fatty acid. J Plant Biochem Biotech 21:51–59

Franklin G, Sheeba CJ, Lakshmi SG (2004) Regeneration of eggplant (*Solanum melongena* L.) from root explants. In Vitro Cell Dev Biol Plant 40:188–191

Gawal NJ, Jarret RL (1991) A modified CTAB DNA extraction procedure for *Musa* and *Ipomoea*. Plant Mol Biol Rep 9:262–266

Gernand D, Golczyk H, Rutten T, Ilnicki T, Houben A, Joachimiak AJ (2007) Tissue culture triggers chromosome alterations, amplification and transposition of repeat sequences in *Allium fistulosum*. Genome 50:435–442

Grabowska-Joachimiak A, Kwolek D, Kula A, Marciniuk P (2012) Fluorescent banding pattern and species-specific DNA marker in *Rumex thyrsiflorus* Fingerh. Cytogenet Genome Res 137:70–77

Jach M, Przywara L (2000) Somatic embryogenesis and organogenesis induced on the immature zygotic embryos of selected sunflower (*Helianthus annuus* L.) genotypes. Acta Biol Crac Ser Bot 42(2):83–86

Kaul S, Das S, Srivastava PS (2013) Micropropagation of *Ajuga bracteosa*, a medicinal herb. Physiol Mol Biol Plants 19(2):289–296

Kengkarj P, Smitamana P, Fujime Y (2008) Assessment of somaclonal variation in Chrysanthemum (*Dendranthema grandiflora* Kitam.) using RAPD and morphological analysis. Plant Tissue Cult Biotech 18(2):139–149

Korpelainen H (2002) A genetic method to resolve gender complements investigations on sex ratios in *Rumex* acetosa. Mol Ecol 11:2151–2156

Kwolek D, Joachimiak AJ (2011) Seed sexing revealed female bias in two *Rumex* species. Acta Soc Bot Pol 80(2):93–97

Lajter I, Zupkó I, Molnár J, Jakab G, Balogh L, Vasas A, Hohmann J (2013) Antiproliferative activity of Polygonaceae species from the carpathian basin against human cancer cell lines. Phytother Res 27:77–85

Litvinenko Y, Muzychkina RA (2008) New antioxidant phytopreparation from *Rumex thyrsiflorus* roots. Chem Nat Compd 44(2):239–240

Ma G, Lu J, Teixeira da Silva JA, Zhang X, Zhao J (2011) Shoot organogenesis and somatic embryogenesis from leaf and shoot explants of *Ochna integerrima* (Lour). Plant Cell Tiss Organ Cult 104:157–162

Mallón R, Rodríguez-Oubiña J, González ML (2011) Shoot regeneration from in vitro-derived leaf and root explants of *Centaurea ultreiae*. Plant Cell Tiss Organ Cult 106:523–530

Mariotti B, Manzano S, Kejnovsky E, Vyskot B, Jamilena M (2009) Accumulation of Y-specific satellite DNAs during the evolution of *Rumex acetosa* sex chromosomes. Mol Genet Genomics 281:249–259

Mizia P, Kwolek D, Ilnicki T (2014) DNA stability contrasts with chromosome variability in *Allium fistulosum* calli. Acta Biol Crac Ser Bot 56(1):66–72

Mosiołek M, Pasierbek P, Malarz J, Moś M, Joachimiak A (2005) *Rumex acetosa* Y chromosomes: constitutive or facultative heterochromatin? Folia Histochem Cytobiol 43:161–167

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15:437–497

Murch SJ, Saxena PK (2001) Molecular fate of thidiazuron and its effects on auxin transport in hypocotyls tissues of *Pelargonium* × *hortorum* Bailey. Plant Growth Regul 35:269–275

Murthy BNS, Murch SJ, Saxena PK (1998) Thidiazuron: a potent regulator of in vitro plant morphogenesis. In Vitro Cell Dev Biol Plant 34:268–276

Namasivayam P, Skepper J, Hanke D (2006) Identification of a potential structural marker for embryogenic competency in the *Brassica napus* spp. *oleifera* embryogenic tissue. Plant Cell Rep 25:887–895

Navajas-Peréz R, De La Herran R, Jamilena M, Lozano R, Ruiz Rejón M, Garrido-Ramos MA (2005) Reduced rates of sequence evolution of Y-linked satellite DNA in *Rumex* (Polygonaceae). J Mol Evol 60:391–399

Navajas-Peréz R, Schwarzacher T, De La Herran R, Ruiz Rejón C, Ruiz Rejón M, Garrido-Ramos MA (2006) The origin and evolution of the variability in a Y-specific satellite-DNA of *Rumex acetosa* and its relatives. Gene 368:61–71

Neelakandan AK, Wang K (2012) Recent progress in the understanding of tissue culture-induced genome level changes in plants and potential applications. Plant Cell Rep 31:597–620

Park SY, Ahn JK, Lee WY, Murthy HN, Paek KY (2005) Mass production of *Eleutherococcus koreanum* plantlets via somatic embryogenesis from root cultures and accumulation of eleutherosides in regenerants. Plant Sci 168:1221–1225

Parker JS, Clark MS (1991) Dosage sex-chromosome systems in plants. Plant Sci 80:79–92

Parveen S, Shahzad A (2011) A micropropagation protocol for *Cassia angustifolia* Vahl. from root explants. Acta Physiol Plant 33:789–796

Pilarska M, Popielarska-Konieczna M, Ślesak H, Kozieradzka-Kiszkurno M, Góralski G, Konieczny R, Bohdanowicz J, Kuta E (2014) Extracellular matrix surface network is associated with non-morphogenic calli of *Helianthus tuberosus* cv. Albik produced from various explants. Acta Soc Bot Pol 83(1):67–73

Popielarska-Konieczna M, Kozieradzka-Kiszkurno M, Świerczyńska J, Góralski G, Ślesak H, Bohdanowicz J (2008) Ultrastructure and histochemical analysis of extracellular matrix surface network in kiwifruit endosperm-derived callus culture. Plant Cell Rep 27(7):1137–1145

Popielarska-Konieczna M, Bohdanowicz J, Starnawska E (2010) Extracellular matrix of plant callus tissue visualized by ESEM and SEM. Protoplasma 247(1–2):121–125

Qin Y, Gao LH, Pulli S, Guo YD (2006) Shoot differentiation, regeneration of cauliflower and analysis of somaclonal variation by RAPD. Hereditas 143:91–98

R Development Core Team (2014) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, http://www.R-project.org/

Razaq M, Heikrujam M, Chetri SK, Agrawal V (2013) *In vitro* clonal propagation and genetic fidelity of the regenerants of *Spilanthes calva* DC. using RAPD and ISSR marker. Physiol Mol Biol Plants 19:251–260

Rychlewski J, Zarzycki K (1986) Genetical and ecological mechanisms regulating the sex ratio in populations of *Rumex thyrsiflorus* Fingerh. (Polygonaceae). Bull Geobot Inst ETH 87:132–140

Sakamoto KL, Shimomura K, Komeda Y, Kamada H, Satoh S (1995) A male associated DNA sequence in a dioecious plant, *Cannabis sativa L.* Plant Cell Physiol 36:1549–1554

Shahzad A, Faisal M, Anis M (2007) Micropropagation through excised root culture of C*litoria ternatea* and comparison between in vitro regenerated plants and seedlings. Ann Appl Biol 150:341–349

Sharma KK, Yeung EC, Thorpe TA (1993) Histology of shoot bud ontogeny from seedling root segments of *Brassica napus* L. Ann Bot 71:461–466

Ślesak H, Liszniańska M, Popielarska-Konieczna M, Góralski G, Śliwińska E, Joachimiak AJ (2014) Micropropagation protocol for the hybrid sorrel *Rumex tianschanicus* × *Rumex patientia*, an energy plant. Histological, SEM and flow cytometric analyses. Ind Crops Prod 62:156–165

Sudha CG, Seeni S (2001) Establishment and analysis of fast-growing normal root culture of *Decalepis arayalpathra*, a rare endemic medicinal plant. Curr Sci 81:371–374

Vinocur B, Carmi T, Altman A, Ziv M (2000) Enhanced bud regeneration in aspen (*Populus tremula* L.) roots cultured in liquid media. Plant Cell Rep 19:1146–1154

Żuk J (1963) An investigation on polyploidy and sex-determination within the genus *Rumex*. Acta Soc Bot Pol 32:5–72