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IN VITRO POLYPLOIDIZATION OF *AJUGA REPTANS* L. USING ORYZALIN

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We have developed an effective protocol for *in vitro* micropropagation in order to obtain large numbers of identical plants and another protocol for *in vitro* polyploidization of *Ajuga reptans*, based on the use of oryzalin. Two donor plants of *A. reptans* (AR 4, AR 7) were treated with 0, 1, 5, 10 μM oryzalin for 2 weeks. The analysis of the ploidy level of these plants was verified by flow cytometric analysis using the internal standardization method. The effects of polyploidization on growth as well as morphological and stomatal size were also measured. After *in vitro* polyploidization, some plants became tetraploids or octoploids. The most efficient conditions for inducing tetraploidy were the treatments with 10 μM oryzalin.

Keywords: *Ajuga reptans*, flow cytometry, micropropagation, oryzalin, polyploidization

INTRODUCTION

Plants of the genus *Ajuga* produce many biological compounds belonging to the chemical groups of alkaloids, anthocyanins, tannins, flavonoids, clerodane, diterpenoids, essential oils, etc. (Mamadalieva et al., 2013; Sivanesan et al., 2016). *Ajuga* plants have anticancer, antioxidant, and antifungal properties (Sadati et al., 2012) and are used in traditional medicines (Park et al., 2017). Micropropagation has become an effective method of asexual propagation; it permits the production of numerous plants from a single individual throughout the year (Park et al., 2017). The studies by Jan et al. (2014) and Sivanesan et al. (2016) demonstrated *in vitro* micropropagation of *A. bracteosa* and *A. multiflora*. Polyploidization demonstrates its important role in genetic and phenotype diversity as well as plant evolution (Alix et al., 2017) and breeding. Karyotype characteristics can provide valuable information on genome evolution and speciation (Xing et al., 2011; Winterfeld et al., 2018). Artificial polyploidy can also enhance the production of secondary metabolites (Tavan et al., 2015). In modern research, for *in vitro* induction of polyploids, the herbicide oryzalin (3,5-dinitro-N_4N_4-dipropylsulfanilamide) is preferred, due to the lower concentrations needed than with colchicine (Xing et al., 2011). This study was aimed to: 1) develop an efficient protocol for *A. reptans* micropropagation; 2) induce polyploid plantlets regeneration using oryzalin; 3) confirm the polyploid level of regenerants.

MATERIAL AND METHODS

PLANT MATERIALS

The plants (AR 4, AR 7) were obtained from the Crop Research Institute (CRI), Prague, Czech Republic. Nodal segments of stolons (2 cm long) were isolated from donor plants, the surface was sterilized and they were planted on MS medium in Petri dishes (60 mm, 1 explant), then after 2 weeks proliferating nodal stolons were used for experiments.

MICROPROPAGATION AND *IN VITRO* POLYPLOIDIZATION (IVP)

One part of the proliferating shoots was subcultured on MS medium 1C; containing 0.1 mg/l indole-3-butyric acid (IBA) + 0.1 mg/l 6-benzyladenine (BA) and another part was passaged on MS medium 1L; containing 0.1 mg/l IBA + 0.5 mg/l BA. Prior to initiating the IVP experiments, the plants were passaged onto MS medium OK containing 0.01 mg/l IBA + 0.01 mg/l BA + 20 mg/l ascorbic acid.

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IN VITRO POLYPLOIDIZATION

A stock solution of oryzalin was prepared according to Greplová et al. (2009). The shoots were placed (4–5 shoots) on hormone-free MS medium, supplemented with oryzalin at concentrations of 0, 1, 5 and 10 μM (2 weeks, thermostat, 25°C) and then transferred on OK medium in tubes and subcultured every 4 weeks. The experiment was repeated twice.

FLOW CYTOMETRIC ANALYSIS

The ploidy level of the *in vitro* regenerated plantlets was evaluated by flow cytometry. *In vitro* samples were prepared according to the procedure described by Doležel et al. (2007). The relative fluorescence intensity of at least 3,000 particles was measured using a Partec CyFlow ML (Partec GmbH, Münster, Gemany) equipped with an argon ion laser (488 nm) and the data obtained were evaluated using FloMax Software, Version 2.9.

MICROPROPAGATION, *IN VITRO* ROOTING OF SELECTED PLANTLETS (CONTROLS, TETRAPLOIDS AND OCTOPLOIDS), MORPHOLOGY AND STOMATAL SIZE

Polyploid and diploid plantlets (control) were propagated on OK medium in tubes and passaged every 5 weeks. The shoots were rooted on hormone-free MS medium and they were transferred to non-sterile conditions (greenhouse, field conditions). The selected plantlets (2x, 4x, and 8x) were chosen for morphological analysis (shape, color and size of leaves) and measurements of stomata size. Samples of epidermal cells were prepared according to Tavan et al. (2015). The stomata were observed with a Zeiss Axio Imager (obj. C-Apochromat 40×/N.A. 0.75, D512 camera 12MPx, Zeiss, Göttingen, Germany).

RESULTS AND DISCUSSION

MICROPROPAGATION

The plants were cultured on 1C and 1L media to avoid vitrification and callus formation. Every 6 weeks of cultivation the plants were separated. More shoots/per explant (4.5) were induced on 1L medium than on 1C medium (2.5/explant).

IN VITRO POLYPLOIDIZATION AND FLOW CYTOMETRIC ANALYSIS

The method of polyploidization involves copies of existing genes and for obtaining polyploid plants, the choice of suitable anti-mitotic agents, appropriate concentration and an optimized protocol play an important role (Dhooghe et al., 2011). The efficiency of polyploidization of the two genotypes was evaluated using flow cytometric analysis. For genotype AR 4, three plantlets were detected as tetraploids – two (28.57%) plants at an oryzalin concentration of 1 μM and one (14.29%) plant at 10 μM. The data are summarized in Table 1. The second genotype (AR 7) showed a higher number of polyploids; a total of ten (47.6%) plantlets have been affected, becoming tetraploids; the most efficient concentration was 10 μM of oryzalin, at which we obtained two octoploid plantlets (Table 1). The flow cytometric

TABLE 1. The effect of *in vitro* oryzalin treatments on induction of polyploidy in *Ajuga reptans* AR 4 and AR 7.

Genotype	Oryzalin μM	Number of plants	Surviving plants		Flow cytometry					
			Number	%	2x	%	**4x**	%	**8x**	%
AR 4	0	10	10	100	10	100	**0**	0	0	0
	1	10	7	70	5	71.43	**2**	28.57	0	0
	10*	10	7	70	6	85.71	**1**	14.29	0	0
AR 7	0	17	17	100	17	100	**0**	0	0	0
	1	17	9	52.94	6	66.66	**3**	33.33	0	0
	5	7	3	42.86	2	66.66	**1**	33.33	0	0
	10*	17	9	52.94	1	11.11	**6**	66.66	**2**	22.22
Treatedplants		61	35	57.38	20	57.14	**13**	37.15	**2**	5.71
Controlplants		27	27	100	27	100	**0**	0	**0**	0

* During two months of cultivation, the surviving plants were smaller than plants in variant 1 μM.

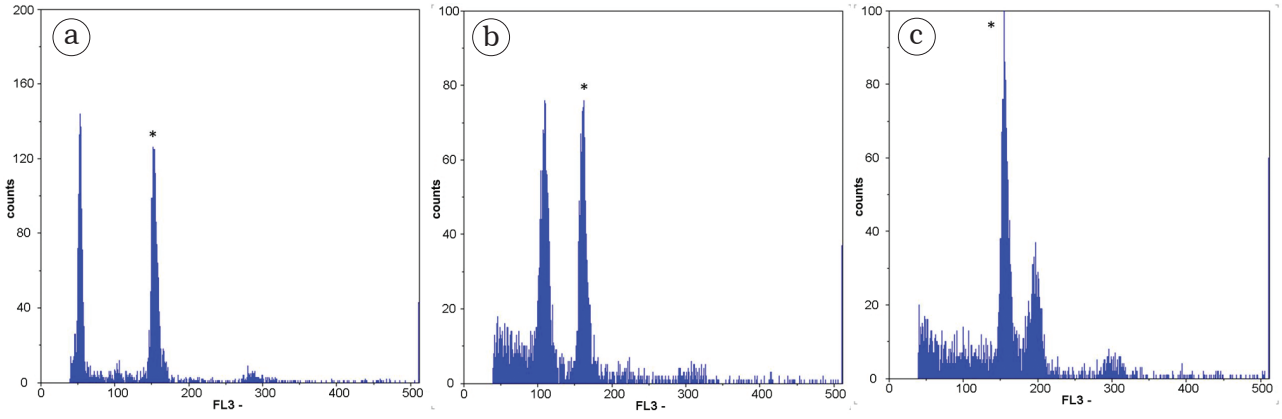

Fig. 1. Representative flow cytometric histograms of leaf DNA contents of *Ajuga reptans* grown under *in vitro* conditions. (**a**) Mixture of diploid control plant not treated with oryzalin and standard*. (**b**) Mixture of tetraploid and standard*. (**c**) Mixture of octoploid and standard*.

histogram (Fig. 1) documents the relative DNA of *A. reptans* and the second peak represents the internal standard *Pisum sativum* cv. Ctirad (2C = 8.76 pg DNA). Viehmannová et al. (2012) polyploidized *Ullucus tuberosus* using oryzalin with a polyploidization efficiency of 11.11% and demonstrated that all the obtained polyploids were octoploids. Greplová et al. (2009) compared the efficacy of colchicine and oryzalin for *in vitro* polyploidization of some wild *Solanum* species. Octoploids induced during *in vitro* culture of *A. reptans* after chemical treatment have never been described till now.

IN VITRO ROOTING OF SELECTED PLANTLETS (CONTROLS, TETRAPLOIDS AND OCTOPLOIDS), MORPHOLOGICAL AND STOMATAL CHARACTERIZATION

The shoots were rooted onto hormone-free MS medium; an average of 95% of the plants already had 3–5 roots after 5 weeks in culture. Diploid and tetraploid plants spontaneously formed roots and fewer differences in the formation of roots and their length were observed, but we saw a bigger contrast in octoploid plants, where the roots were very short (Fig. 2a). The rooted plants of the

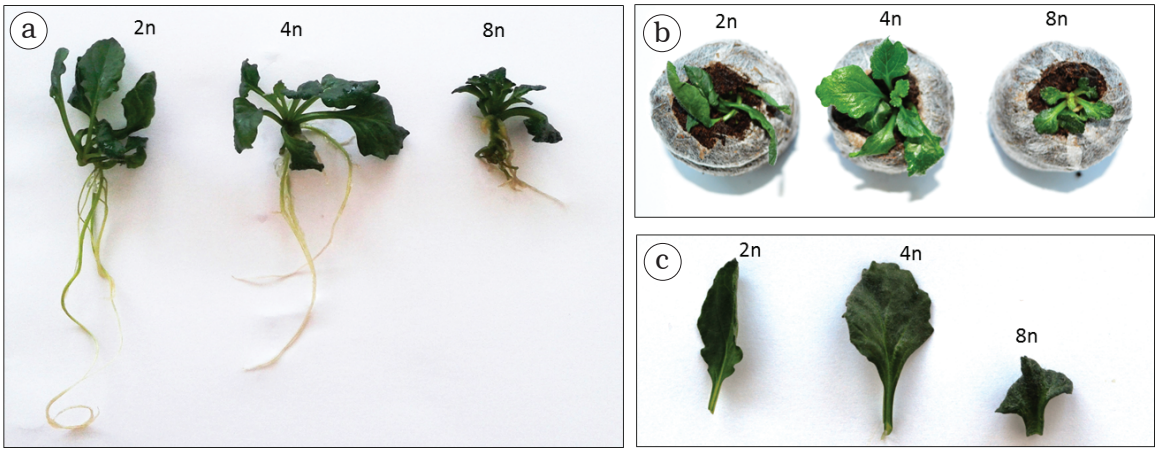

Fig. 2. Comparison of the morphology of control and polyploid plants. (**a**) Rooting of polyploids. (**b**) Converted plants in the Jiffy pellets. (**c**) Variation in leaf shape of *A. reptans*.

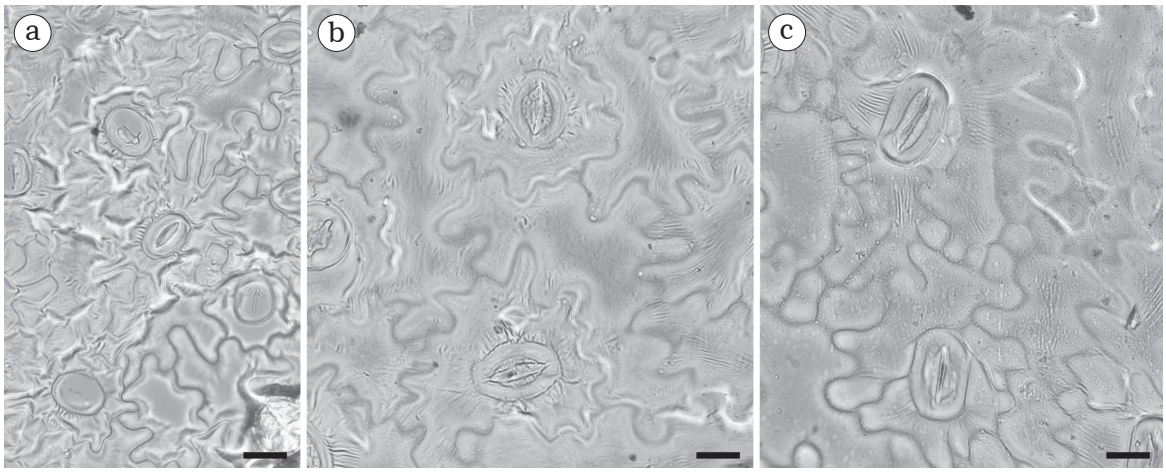

Fig. 3. Stomata size in *Ajuga reptans*. (**a**) Diploid control plant. (**b**) Tetraploid plant. (**c**) Octoploid plant. Scale bar = 20 μm

regenerated subclones and control plants were transferred to non-sterile conditions (Fig. 2b). All obtained polyploid plantlets had shorter petioles and broader – rounded leaf blades (Fig. 2c) and formed roots more slowly. We can consider that the ploidy level is an important factor affecting the leaf shape. The size of stomata in the tetraploids and octoploids was significantly larger than in the diploid control plants (Fig. 3). Xing et al. (2011) demonstrated important differences in stomata size and density in tetraploids of *Catharanthus roseus* L. after treatment with colchicine.

CONCLUSION

In this paper, we demostrated an appropriate protocol for the *A. reptans* micropropagation, *in vitro* polyploidization using oryzalin and procedure for micropropagation of polyploid plantlets together with their conversion to *ex vitro* and field conditions. We suppose that chromosome doubling can be an effective approach to enhancing production of secondary metabolites and may become an effective tool for medicinal purposes.

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