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MŠ: wrote the manuscript, performed flow cytometry analysis; BN: designed experiments, wrote the manuscript, performed micropropagation and oryzalin treatment; PH: performed stomata analysis; VO: provided critical feedback and revised the manuscript, performed chromosome counting

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ORIGINAL RESEARCH PAPER

Artificial induction of tetraploidy in *Humulus lupulus* L. using oryzalin

Michaela Švécarová, Božena Navrátilová, Petr Hašler, Vladan Ondřej*

Department of Botany, Faculty of Science, Palacký University, Šlechtitelů 27, 783 71 Olomouc, Czech Republic

* Corresponding author. Email: vladan.ondrej@upol.cz

Abstract
The aim of the research was to establish an efficient procedure for in vitro micropropagation in order to obtain numbers of identical plants for in vitro polyploidization of *Humulus lupulus* ($2n = 20$), using antimicrotubule agent oryzalin. For this purpose, the polyploidization was carried out for *H. lupulus* Osvald's clones 31, 74, 114, and for 'Sladek' cultivar. The two experimental methods – the cultivation of nodal segments on medium with different concentrations of oryzalin (1, 5, and 10 µM) for 2 weeks and the irrigation of nodal segments with oryzalin (10 and 20 µM) for 24 and 48 h were chosen for inducing for polyploid plantlets of *H. lupulus*. This procedure provided tetraploids, which were identified by flow cytometry using internal standardization method and confirmed using chromosome counting of methaphasic cells from the root tips and morphological observations. The influence of chromosome doubling was also verified with stomata characterization. The polyploid plants were propagated for next evaluation, rooting and transfer to nonsterile conditions and into field. After chromosome doubling, using some different concentration of oryzalin, some plantlets became tetraploids, no mixoploids were detected. The highest efficiency of polyploidization was achieved for clone 72 after 2-week treatment of oryzalin supplemented medium. On the other hand, for method based on the irrigation of nodal segments with oryzalin, the most efficient conditions were treatment with 10 µM and 20 µM oryzalin for 24 and 48 h, respectively.

Keywords
Cannabaceae; *Humulus lupulus*; flow cytometry; karyology; micropropagation; oryzalin; tetraploidy

Introduction

Hop (*Humulus lupulus* L.) is a perennial species in the Cannabaceae family and is widely cultivated throughout the temperate zones of the world. About 80,000 ha are in commercial production worldwide [1]. Only female plants that produce inflorescences (called cones) are used for productive plantations. Male plants which grow in the vicinity need to be removed to prevent the pollination of female inflorescences and the production of seeds in the cones [2]. The cones provide a valuable raw material rich in chemical compounds important for the brewing industry [3,4]. Profiles of chemical components in hop cones are dependent on the cultivar and the health condition of plants. Hop is still used in the pharmaceutical and cosmetic industries and was traditionally employed in herbal medicines, especially for the treatment of sleeping disorders, as a mild sedative and for the activation of gastric function as bitter stomachic [5]. Hops contain two groups of flavoring compounds produced by the lupulin glands in the female plant. These soft resins containing the bittering precursors are used for beer and essential oils that include many of the aromatic constituents used in brewing [6]. Hop cones are also a valuable source of bioactive substances such as xanthohumol which exhibits a strong antioxidant activity and can inhibit tumor formation [7]. The

main groups of the secondary metabolites are polyphenols (catechin, rutin, coumarin, quercetin, and others), alpha and beta bitter acids (humulone, lupulone, etc.), and essentials oils [8].

Micropropagation of various plant species including many medicinal plants is described in a wide range of scientific studies [9], and is a useful method for the multiplication of selected genotypes and chemotypes. It has become an effective or alternative method for asexual propagation, practicable within short time periods and with limited space demands; it permits the rapid production of numerous plants from a single individual [10]. Several publications describe the procedures of in vitro micropropagation of *H. lupulus* [8,11,12]. Polyploids can be induced by two mechanisms, mitotic polyploidization, which is based on the doubling of chromosomes in somatic tissues, and meiotic polyploidization, which generates 2*n* gametes [13]. The first application of mitotic polyploidization in in vitro cultures (tobacco) was made by Murashige and Nakano in 1966 [14]. The traditional method to obtain polyploid plants is to use colchicine which is an alkaloid contained in seeds and bulbs of *Colchicum autumnale* L. [15]. In vitro chromosome doubling can also be induced by several antimitotic agents including oryzalin, nitrous oxide, amiprophos methyl, and trifluralin [16,17]. Some studies have described oryzalin to be a more efficient agent than colchicine and refer to it as a preferable alternative to using the very toxic colchicine [18]. For example, DNA doubling can be achieved with alternative mitotic inhibitors (e.g., oryzalin, etc.) at about 100–1,000 times lower concentrations than with colchicine [19]. Oryzalin (3,5-dinitro-N_4N_4-dipropylsulfanilamide) is a dinitroaniline herbicide with strong antimitotic activity which can be used for the induction of polyploidy; it binds to tubulin and inhibits polymerization of microtubules leading to chromosome doubling. The production of hop tetraploids was reported by Roborgh (1969) and Haunold (1972) (cited by [3]). The basic chromosome number of *H. lupulus* is 10, with diploids ($2n = 2x = 20$) being found in nature [20]. Polyploid plants contain more than two complete sets of chromosomes and are heritable. Roy et al. [11] have shown in vitro tetraploid induction in hop using colchicine. Similar works [3,5] have described the procedure of in vitro polyploidization in *H. lupulus*. The effective application of the herbicide oryzalin has been realized in *Gerbera* [18], *Lilium* [21], potato [22], *Buddleja* [23], *Hypericum* species [24], *Alocasia* [25] and *Dracaena deremensis* [26]. The pre-eminent method for evaluation of the induced polyploidization is flow cytometry. Additional confirmatory methods are chromosome counting and morphological or anatomical observations [14].

We report here a method for in vitro micropropagation to develop tetraploid plantlets of *H. lupulus*, and appropriate protocols for generating polyploid plants using oryzalin. Flow cytometric analysis (FCM) was used for checking the ploidy level of the treated plantlets. Other methods such as chromosome counting and measurement of stomatal length and morphology were also employed, and the results obtained were verified using diploid control plants.

Material and methods

Plant material

In vitro plantlets of *H. lupulus* Osvald's clones Nos. 31, 72, 114, and 'Sladek' cultivar were obtained from the Hop Research Institute Co. Ltd. (Zatec, the Czech Republic).

In vitro micropropagation

The plantlets were cultivated on Murashige–Skoog (MS) medium supplemented with 0.01 mg L^{-1} IBA (indole-3-butyric acid) (0.049 μM), 0.01 mg L^{-1} BA (6-benzyladenine) (0.044 μM), 30 g L^{-1} sucrose, and 0.8% agar in De Wit culture tubes (Duchefa, Haarlem, the Netherlands). Cultures were maintained at 22 +2°C, 16/8 h day/night. They were subcultured repeatedly every 5 weeks and cut into segments with three pairs of leaves to obtain a sufficient number of plantlets for experiments with oryzalin.

Oryzalin treatment

Nodal segments of three actively growing clones of *H. lupulus* (Nos. 31, 72, and 114) and of 'Sladek' were chosen for in vitro induction of polyploid plants. Nodal segments (third to fifth from the apex of donor plants) with one leaf were used as explants. A stock solution of oryzalin (Sigma-Aldrich, St. Louis, MO, USA) was prepared according to Greplová et al. [27] and the respective concentrations of oryzalin for the experiments were prepared from this stock solution.

The first method was cultivation of nodal segments with oryzalin present in the medium for 2 weeks (CMNS). The nodal segments obtained (9–12 of all samples) were placed in Erlenmeyer flasks on hormone-free MS medium containing 0, 1, 5, or 10 μM oryzalin and segments cultivated in a thermostatically-controlled room at 25°C for 2 weeks. They were then transferred on to MS medium containing 0.01 mg L^{-1} IBA, 0.01 mg L^{-1} BA, and 20 mg L^{-1} ascorbic acid in De Wit culture tubes (one segment/tube) and subcultured every 4 weeks. Each regenerated segment was taken as one clone. The experiment was repeated three times. The second method chosen was irrigation of nodal segments with oryzalin solution (ONS). Nodal segments (10–15 pieces) were placed in an Erlenmeyer flask on hormone-free MS medium and irrigated for 24 h with 15 mL with different oryzalin solutions of 10 and 20 μM for clones (Nos. 31, 72, and 114) and 10, 20 μM for 'Sladek', as well as in sterile distilled water, as the control. The cultivation of segments was carried out in the thermostatically-controlled room at 25°C. After applying the treatments for 24 h, the oryzalin solution was removed. The nodal segments were washed three times in sterile distilled water and placed on to MS medium with 0.01 mg L^{-1} IBA, 0.01 mg L^{-1} BA, and 20 mg L^{-1} ascorbic acid in Petri dishes for 2 weeks, and then every 4 weeks on the same medium in the culture tubes (one segment/tube). Each shoot generated was taken as one clone. The experiment was repeated twice. After 4–6 months of cultivation, actively growing plants were selected for flow cytometry, chromosome observation, and morphological comparison of the plants obtained, as well as with the control plants (length of nodes, color and size of leaves, rooting).

Micropropagation of polyploid plantlets

The actively-growing shoots gained from the test with oryzalin were verified by flow cytometry and only the polyploids and diploid controls were propagated. Micropropagation of the polyploid plants was achieved on MS medium with 0.01 mg L^{-1} IBA, 0.01 mg L^{-1} BA, and 20 mg L^{-1} ascorbic acid in culture tubes, subculturing every 5–6 weeks.

In vitro rooting of polyploids, morphology and observation of stomata

The shoots of polyploids were rooted on hormone-free MS medium in Erlenmeyer flasks and were grown on in the culture room. Before transferring to nonsterile conditions, the flasks were firstly covered with aluminum foil for 1 week. The plants were then removed from the flasks and the roots were carefully rinsed with running water. The longer roots were next shortened and old leaves excised. Finally, the completed samples were planted in Jiffy pellets, held under foil, and moved to a greenhouse for 6–10 weeks. At the end of this period, the most vigorous plants were planted out into field conditions at locations in Prague and Zatec, the Czech Republic.

The selected plants (diploid controls and tetraploids) were also used for morphological observation (shape, rooting, color and size of leaves) and characterization of the stomata after 1–2-month growth in Jiffy pellets and also those grown under field conditions. For stomatal measurements, specimens of epidermal cells were acquired from leaf surfaces using the nail varnish technique [28]. The number, size, and characteristics of the stomata were observed with a Zeiss Axio Imager [objective C-Apochromat 40×/N.A. 0.75, D512 camera 12 MPx (Zeiss, Göttingen, Germany)].

Flow cytometry analysis of ploidy level and chromosome counting

Ploidy levels of the plants of *H. lupulus* treated with different oryzalin concentrations and by the two methods of induction of polyploidy were determined by FCM. Young leaves of the oryzalin-treated plants were used for measurement of DNA content by flow cytometry, using the method of internal standardization (Fig. 1). One-cm long *H. lupulus* in vitro leaf samples and the same amount of the internal standard *Glycine max* Merr. 'Polanka' (2C = 2.50 pg DNA) [29] were chopped with a sharp razor blade in a glass Petri dish containing 1.5 mL of ice-cold LB01 buffer at pH 7.8, and then filtered through 22-µm nylon mesh. The suspension of nuclei released was stained with 50 µL of DAPI (4′,6-diamino-2-phenylindol) and incubated at room temperature for 10 min. Finally, the relative fluorescence 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). The data obtained were evaluated using FloMax software, version 2.9 and the histograms of the fluorescence intensity were registered over 512 channels.

Root tips were collected and cut from roots of selected plants cultivated in vitro showing FCM-positive as diploids and tetraploids. They were treated with 0.01 mol L^{-1} 8-hydroxyquinoline overnight at 25°C, followed by fixation in an ethanol:glacial acetic acid mixture (3:1), and then stored at 4°C. These materials were washed in 96% ethanol and distilled water and hydrolyzed with 5 mol L^{-1} HCl for 25 min followed by staining with Schiff's reagent. The treated root tips were excised to be 1-mm long and squashed on a glass slide with a drop of 45% (v/v) acetic acid and protected with a cover slip. Chromosome numbers were counted using a light microscope (BX60, Olympus, Optical Co., Tokyo, Japan).

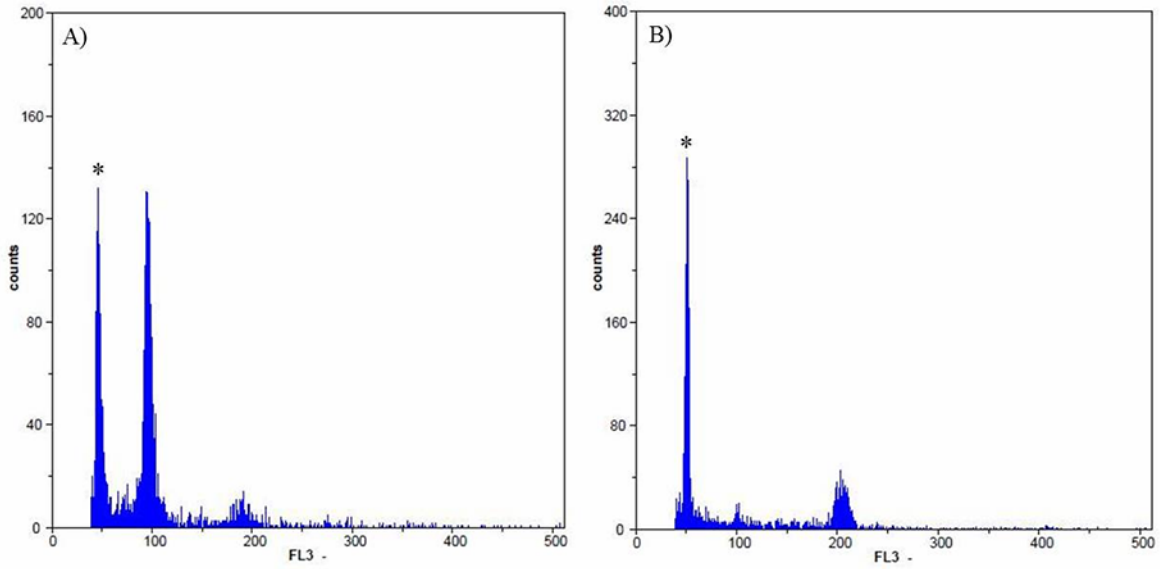

Fig. 1 Representative flow cytometric histograms of leaf DNA content of *H. lupulus* (Clone 72, Sample 25) grown under in vitro conditions and internal *Glycine max* standard. (**A**) Diploid control plants without oryzalin treatment and standard (*). (**B**) Tetraploid and standard (*).

Results

In vitro polyploidization, flow cytometric analysis, and karyology

In this study, a total of 203 nodal segments of clone No. 31 (171 oryzalin influenced, 32 as a control), 185 of nodal segments of clone No. 72 (147 oryzalin influenced, 38 as a control), 191 of nodal segments of clone No. 114 (164 oryzalin influenced, 27 as a control), and 168 of nodal segments of *H. lupulus* 'Sladek' (140 oryzalin influenced, 28 as a control) were used for artificial induction of polyploids using oryzalin. In these experiments, three clones of hop ('Sladek') and two methods of in vitro polyploidization were compared. The first method was CMNS (treatment with 1, 5, and 10 µM oryzalin

for 2 weeks), and the second method was ONS (oryzalin treatment with 10 and 20 μM, exposure time 24 or 48 h). These were always compared with the nonoryzalin-influenced controls. The efficiency of polyploidization of all clones and 'Sladek' after treatment with different concentrations of oryzalin for 2 weeks or 24/48 h was evaluated using flow cytometric analysis, karyology, and morphological or anatomical observation. For Clones 31 and 93, nodal segments were used in the cultivation on oryzalin medium (CMNS) and a total of 32 surviving plants were obtained. Fourteen plantlets (43.75%) treated with oryzalin (2 weeks) were detected as tetraploids. The most efficient condition was cultivation for 2 weeks on the medium supplemented with oryzalin (5 μM). For the ONS method, the highest efficacy was when using 20 μM oryzalin for 48 h. The total number of tetraploid plantlets obtained was seven (58.33%).

We examined Clones 72 and 114 and compared the two methods (CMNS and ONS). The greatest yield of tetraploid plantlets was achieved for clone No. 72. The total number of nodal segments in the CMNS experiment was 88 and 31 surviving plants (35.23%) were obtained, of which 17 were tetraploid (54.84%). The greatest number (3; 100%) of tetraploid plants in the ONS test for clone No. 72 was obtained with the 10 μM treatment of oryzalin and an exposure time of 24 h. Forty-three percent of surviving plantlets of clone No. 114 were obtained after cultivation on oryzalin medium. For this clone, 10 plants became tetraploids (23.26%), four (30.77%) at a concentration of oryzalin of 1 μM, three (21.43%) at 5 μM, and also three (25%) at 10 μM. In the case of the ONS method, we detected a total of five tetraploid plants, two tetraploids after 10 μM oryzalin treatment for 24 h and three tetraploids at 20 μM oryzalin for 48 h, respectively. From these results, we can assert that the CMNS method is much more effective than the ONS. The data obtained demonstrate that the polyploidy efficacy for three clones of *H. lupulus* (Osvald's clones) is the greatest for clone No. 72 for the CMNS method, and the most efficient concentration was 10 μM oryzalin. The well known 'Sladek' cultivar was also tested by CMNS, and for 102 plants, 29 plantlets survived and only five (17.24%) plantlets from these survivors became tetraploids. The most efficient concentration was 5 μM of oryzalin. The use of ONS methods is clearly inappropriate for 'Sladek' as we obtained no plants after oryzalin treatment. The induction of polyploidy of *H. lupulus* clones Nos. 31, 72, and 114 (Osvald's clones) had a greater yield than did polyploidization for 'Sladek'. For all samples in our study we detected tetraploid plants, but no mixoploids were found. All data from the outcomes of the CMNS and ONS methods are summarized in Tab. 1 and Tab. 2.

Our data from the flow cytometry were also supported by chromosome counts and morphological characterization. The numbers of chromosomes in diploid and tetraploid samples are summarized in Fig. 2.

In vitro growth, rooting of polyploid plants, and morphological and stomatal characterization

Within several months of exposure to oryzalin the growth of the regenerants was noticeably slower than that of the controls plants. Four months after oryzalin treatment, most of tetraploid plantlets were smaller (only 1–2 cm) with shorter internodes, a light green or dark green color of the leaves, and were slow rooting (Fig. 3A) compared to the shoots of the controls (8 cm). The latter were green, long, and well rooted, as were the diploid regenerants obtained after oryzalin treatment (which had the same phenotype as the controls and were not cultivated further). Rooted tetraploids obtained by both the CMNS and ONS methods and control plantlets were acclimated and transferred to nonsterile conditions into Jiffy pellets 12 months after oryzalin treatment (Fig. 3B). Only tetraploids and controls are now maintained under field conditions on locality at the Crop Research Institute, Prague, the Czech Republic and in a glasshouse at the Hop Research Institute Co. Ltd., Zatec, the Czech Republic. Plants determined as tetraploids after oryzalin treatment had larger leaf areas than did the control plants (Fig. 3C). We can also confirm that ploidy level is an important factor affecting leaf shape. Moreover, the stomatal size of tetraploid plantlets was greater than that of diploid control plants (Fig. 4). From our results, we can consider that the stomatal dimensions of tetraploids of *H. lupulus* are larger than those of diploids.

Tab. 1 Cultivation of nodal segments of Osvald's clones and 'Sladek' cultivar on oryzalin medium for 2 weeks.

Genotype	Concentration (μM)	Number of nodal segments	Survival (number)	Diploid (number)	Tetraploid (number)
No. 31	1; 5; 10	30; 31; 32	15; 11; 6	11; 5; 2	4; 6; 4
Total	-	93	32	18	14
No. 72	1; 5; 10	30; 27; 31	15; 6; 10	9; 2; 3	6; 4; 7
Total	-	88	31	14	17
No. 114	1; 5; 10	32; 32; 36	17; 14; 12	13; 11; 9	4; 3; 3
Total	-	100	43	33	10
'Sladek'	1; 5; 10	34; 34; 34	14; 13; 2	13; 10; 1	1; 3; 1
Total	-	102	29	24	5

Tab. 2 Irrigation of nodal segments of Osvald's clones and 'Sladek' cultivar with oryzalin solution for 24/48 h.

Genotype	Concentration (μM)	Number of nodal segments	Time of exposure (h)	Survival (number)	Diploid (number)	Tetraploid (number)
No. 31	10; 20; 20	23; 20; 35	24; 24; 48	3; 3; 6	2; 1; 2	1; 2; 4
Total	-	78	-	12	5	7
No. 72	10; 20	32; 27	24; 24	3; 1	0; 0	3; 1
Total	-	59	-	4	0	4
No. 114	10; 20; 20	24; 20; 20	24; 24; 48	3; 0; 4	1; 0; 1	2; 0; 3
Total	-	64	-	7	2	5
'Sladek'	10; 20	17; 21	24; 24	0; 0	0; 0	0; 0
Total	-	38	-	0	0	0

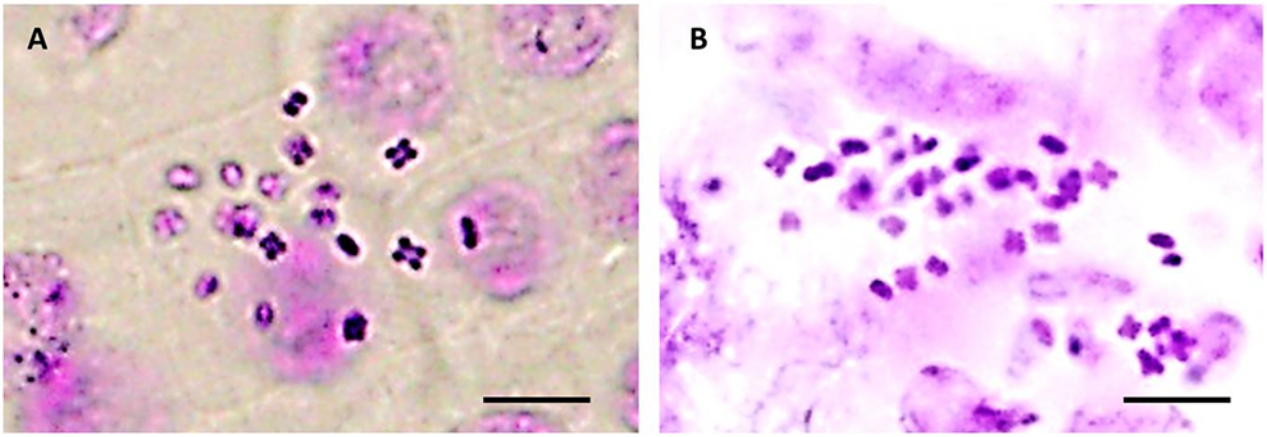

Fig. 2 Chromosome numbers of *H. lupulus* (Clone 72, Sample 25). (**A**) Diploid control plant ($2n = 2x = 20$). (**B**) Tetraploid plant ($2n = 4x = 40$). Scale bar is 5 μm.

Discussion

Production of polyploid forms is one of the conventional methods used in plant breeding. In the case of *H. lupulus*, the production of tetraploids has became the subject of interest in several research papers [3]. Polyploidization (in vivo or in vitro) involves copies of existing genes and can bring about anatomical and morphological changes and can lead to increased production of secondary metabolites. The induction of polyploidization depends on a large number of variables, such as explant type, genotype, the medium used, the antimitotic agents, their time of exposure, and concentrations.

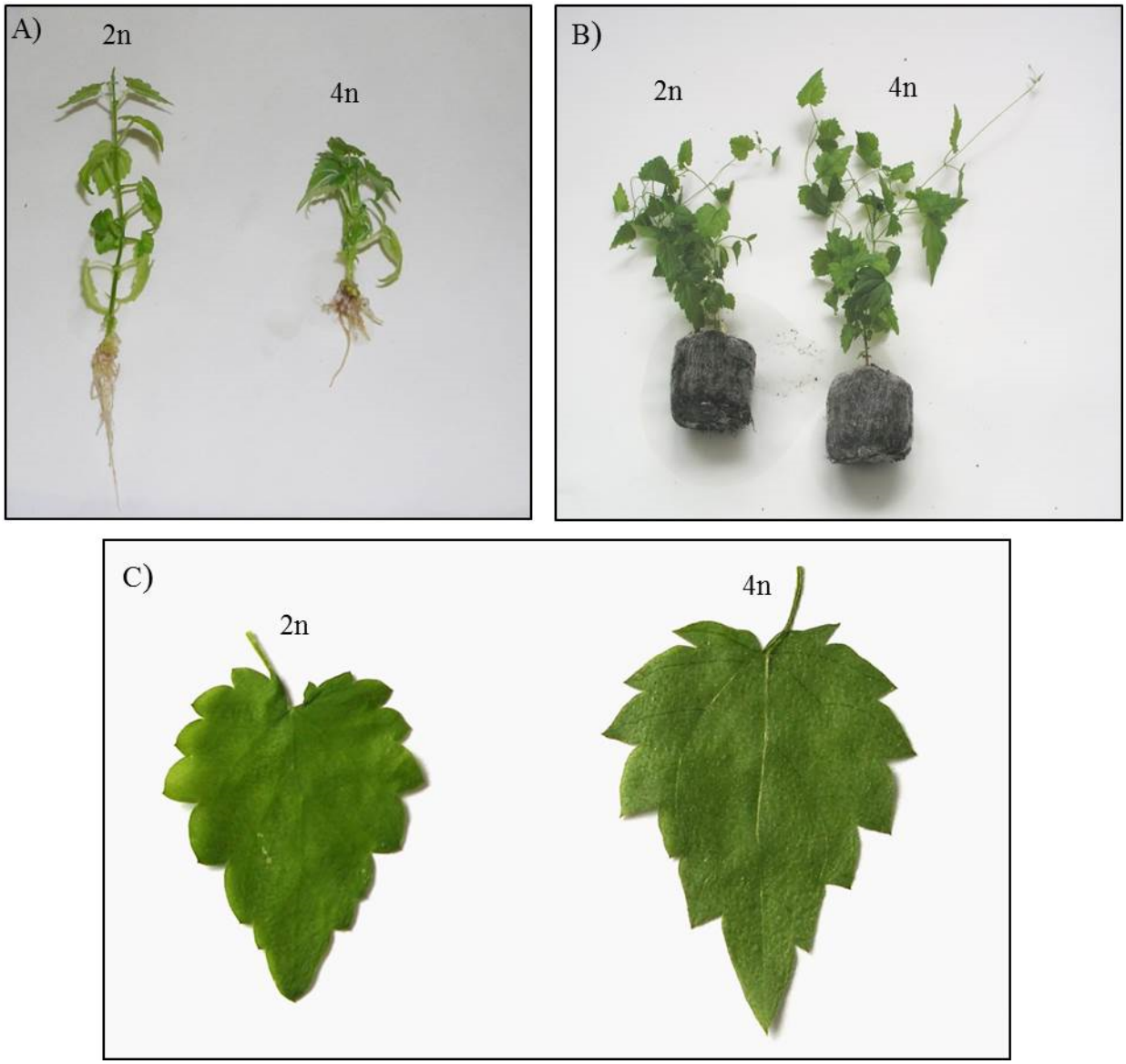

Fig. 3 Comparison of the morphology of control and tetraploid plantlets. (**A**) Rooting of polyploid (Clone 114, Sample 41) and control. (**B**) Converted plants (Clone 114, Sample 41) in the Jiffy pellets and control. (**C**) Variation in leaf shape of *H. lupulus* (Clone 114, Sample 41, and control).

Low doses are not effective, whereas excessively high doses can be lethal [14]. The most used active substances are oryzalin, colchicine, or trifluralin. Successful explant types are plantlets or shoots, buds and shoot tips, callus, somatic or zygotic embryos, seeds, nodal segments, etc.

Škof et al. [30] described an optimal treatment for *H. lupulus* as 0.05% colchicine for 48 h, and they obtained 25.6% of tetraploids. In another paper [8], the highest number of tetraploids of *H. lupulus* was achieved when explants were immersed in 0.05% colchicine for 48 h. Also, in vitro tetraploid induction using colchicine in *H. lupulus* was evaluated by Roy et al. [11] after treatment with 0.05% colchicine for 48 h. In the present study, oryzalin at different concentrations and exposure times is also able to induce tetraploid plants in vitro. Several interesting studies reported results using oryzalin as an antimitotic agent. For example, Meyer et al. [24] examined polyploid induction from leaves of *Hypericum* and the greatest percentage of polyploids was induced with 30 μM oryzalin. Other research has reported chromosome duplication in *Alocasia* [25] where the most efficient condition for inducing tetraploids seemed to be treatment with 0.01% oryzalin for 24 h. Lam et al. [16] describe tetraploid induction in *Acacia crassicapra* using oryzalin; the most effective treatment was exposure to 0.005% oryzalin for 24 h and the tetraploid induction efficiency was 3.10%. Treatment

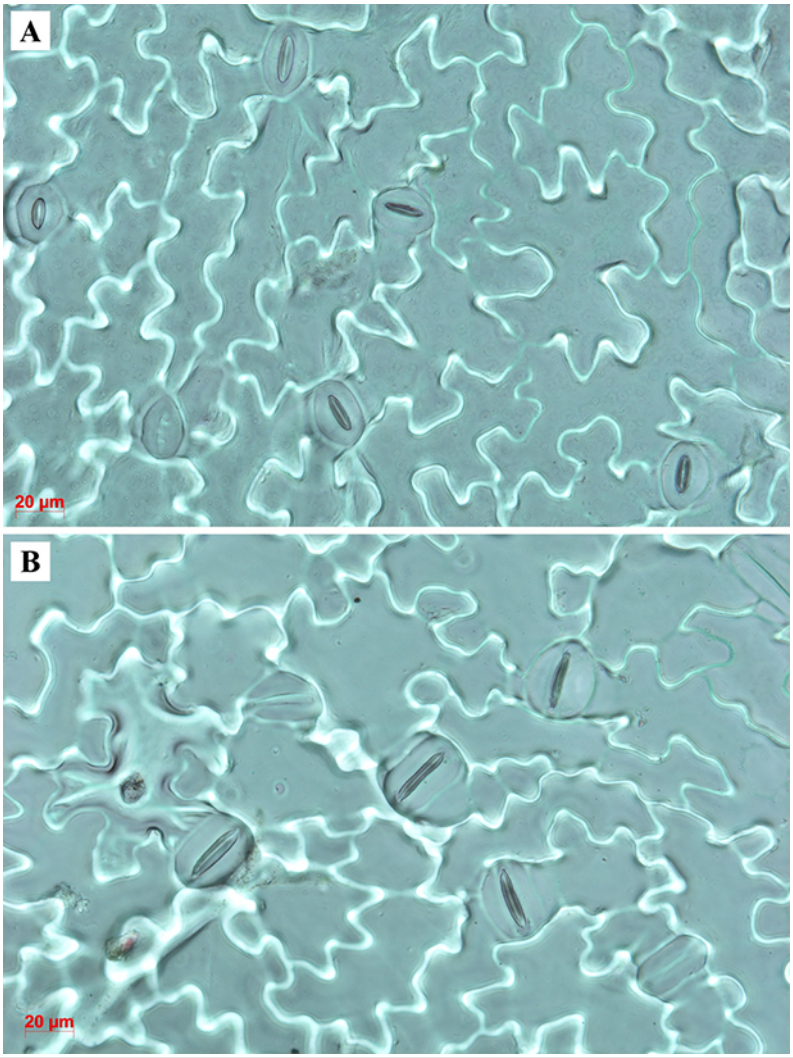

Fig. 4 The morphology of stomata, their size and density in *H. lupulus* (Clone 72, Sample 25). (**A**) Diploid control plant (length 27.7 ±2.16 µm, width 17.61 ±1.85 µm). (**B**) Tetraploid plant (length 37.87 ±3.82 µm, width 23.18 ±2.55 µm). Scale bar is 20 µm.

with oryzalin produced more mixoploids than did colchicine, however in this study, only tetraploids were obtained using oryzalin.

Tetraploid status was revealed by the flow cytometry and also supported by chromosome counts. Trojak-Goluch and Skomra [8] presented a mitotic analysis of *H. lupulus* after colchicine treatment (0.05%, 48 h) and confirmed that cells of nontreated diploid *H. lupulus* contained $2n = 2x = 20$ chromosomes whereas the cells from the confirmed (FCM) tetraploid plants had $2n = 4x = 40$. Tetraploid plants displayed also morphological changes in leaf area, shape, and stomatal size. Thao et al. [25] have stated that ploidy level may be considered to be a major factor affecting variations in leaf shape and display in *Alocasia* after colchicine or oryzalin treatment. Some reports (e.g., [25]) suggest that after in vitro polyploidization stomatal dimensions can be greater. Xing et al. [15] report important differences in stomatal size and density in *Catharanthus roseus* L. after colchicine treatment.

Conclusion

Oryzalin treatment led to the production of tetraploid plantlets of *H. lupulus* and showed its efficacy. Treated plantlets that were tested for their ploidy level by flow cytometric and karyological analyses are new breeding materials with the potential for improvement of flavor chemical components in the commercial hop crop.

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Sztuczna indukcja tetraploidalności chmielu zwyczajnego (*Humulus lupulus* L.) za pomocą oryzaliny

Streszczenie

Celem badań było ustalenie efektywnej metody mikrorozmnażania *in vitro* w celu uzyskania wielu identycznych roślin do poliploidyzacji chmielu zwyczajnego (*Humulus lupulus* L.) (2*n* = 20), z wykorzystaniem związku powodującego degradację mikrotubul – oryzalinę. W tym celu, przeprowadzono poliploidyzację chmielu Osvald, klonów 331, 74, 114 i odmiany 'Sladek'. W celu indukcji polyploidyzacji sadzonek chmielu zastosowano dwie metody eksperymentalne (***i***) uprawa fragmentów węzłowych na pożywce z oryzaliną w różnych stężeniach (1, 5 i 10 μM) przez 2 tygodnie, (***ii***) podlewanie fragmentów węzłowych oryzaliną (10 i 20 μM) przez 24, 48 godzin. Procedury te zapewniły uzyskanie tetraploidów, które zostały zidentyfikowane za pomocą cytometrii przepływowej z wykorzystaniem wewnętrznej standaryzacji, liczenie chromosomów w komórkach z wierzchołków korzeni będących w metafazie, obserwacje morfologiczne oraz charakterystyki aparatów szparkowych. Rośliny poliploidalne były rozmnażane w celu dalszego sprawdzania, ukorzeniania oraz przenoszenia do warunków niesterylnych oraz do gruntu. Po podwojeniu chromosomów z wykorzystaniem różnych stężeń oryzaliny, niektóre sadzonki stały się tetraploidalne, ale nie stwierdzono miksoploidów. Największym współczynnikiem poliploidyzacji został osiągnięty dla klonu 72 po dwutygodniowej uprawie na pożywce zawierającej oryzalinę. Po zastosowaniu metody z nawadnianiem fragmentów węzłowych oryzaliną najbardziej efektywne okazało się traktowanie 10 μM i 20 μM oryzaliną odpowiednio przez 24 lub 48 godzin.