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IN VITRO ORGANOGENESIS IN *RUMEX THYRSIFLORUS* FINGERH. – PROBLEMS OF SEX RATIOS

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Abstract. *Rumex thyrsiflorus* Fingerh. is one of the few dioecious plant species, which have sex chromosomes. We conducted the preliminary experiments to determine the type of morphogenesis of *R. thyrsiflorus* explants cultured *in vitro* and to verify, using PCR-based methods, if there is the relationship between sex and morphogenetic response of explants micropropagated under *in vitro* conditions. The results of our studies revealed the female-biased sex ratios among explants cultured *in vitro* (M:F=1:1.7). The female-biased sex ratios in case of explants showed organogenesis *in vitro* (M:F=1:2.44) may suggest a higher regeneration ability of female explants.

Key words: *Rumex thyrsiflorus, in vitro* culture, organogenesis, histological analysis, SEM, sex chromosomes, sex ratio, genetic sex marker

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Rumex thyrsiflorus Fingerh. is one of the few dioecious plant species, which have sex chromosomes. The chromosome constitution of females is 2n=12A+XX and males is 2n=12A+XY_1Y_2 (Żuk 1963). *R. thyrsiflorus* appeared to be an interesting object of studies on structure and function of chromosomes and sex chromatin and also for studying the sex ratio, a comparison between the primary ratio in seeds and the secondary in populations (Rychlewski & Zarzycki 1986). Although a chromosomal sex determination system is expected to constrain the average primary sex ratios to a 1:1 ratio, the operational sex ratios (the numbers of males per female at sexual maturity) may be biased due to differences between the sexes in germination, mortality, vegetative vigour, flowering frequency, environmental responses, or due to a genetic mechanism distorting the sex ratios (Korpelainen 2002). Biased sex ratios in populations are interesting phenomena observed in many dioecious plants. In some species female specimens predominate, while others are male-biased (Błocka-Wandas *et al.* 2007, and references therein).

We conducted the preliminary experiments to verify the type of morphogenetic response of *R. thyrsiflorus* explants cultured *in vitro* and to examine sex ratio among all explants used and explants with morphogenetic potential. We wanted to verify, using PCR-based methods, if there is the relationship between sex and morphogenetic response of explants micropropagated under *in vitro* conditions.

Histological and SEM analysis

During experiments the hypocotyls isolated from 11-day-old seedlings were used as explants. They were cultured on the media supplemented with different concentration of following plant growth regulators: 2,4-D, BAP and TDZ. For histological analysis the material was prepared for embedding tissues in Technovit 7100 as it was described by Ślesak *et al.* (2013), sectioned to 5 μm with a rotary microtome and stained using periodic acid Schiff/naphthol blue black (PAS/NBB) double staining.

The callogenesis was observed on all cultured explants, irrespective of their sex. Callus tissue was heterogenous and composed of cells varied in shape, size and vacuolization degree. Large, highly vacuolated callus cells were loosely attached, contrary to small, isodiametric cells with dense cytoplasm forming meristematic centres on the surface (2,4-D, BAP, TDZ) and also in the internal region of the callus (TDZ).

Numerous starch grains were visible firstly in cortex cells and subsequently in stele cells. Histological and scanning electron microscope (SEM) analysis revealed that the regeneration of plantlets occurred *via* indirect organogenesis (adventitious shoots formation *via* callus). The first signs of morphogenetic response were visible on all tested culture media about ten days from the beginning of the culture. Secondary organogenesis was also observed.

Sex ratio analysis

To analyze sex ratio among explants of *R. thyrsiflorus* cultured *in vitro*, PCR-based methods, involving DNA markers located on Y chromosomes were used. DNA was extracted from cultured explants by CTAB method (Gawal & Jarret 1991) with modifications (Kwolek & Joachimiak 2011). The following primers were used: UGR08-F and UGR08-R, primers specific for the male-specific repetitive sequence RAYSII in *R. acetosa* L. (Mariotti *et al.* 2009). 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) occurred in all analyzed male plants. They also had an additional amplification product with a size of around 600 bp. The shorter fragment may be a potentially useful molecular marker for taxonomical and population genetic studies on *R. thyrsiflorus* and its hybrids (Grabowska-Joachimiak *et al.* 2012). None of these products occurred in female plants.

We also confirmed, likewise Kwolek & Joachimiak (2011), the usefulness of the RAY-f and RAY-r primers, developed by Korpelainen (2002). These primers amplifying the male-specific RAYSI sequence presents on the Y chromosomes of *R. acetosa* and its close relatives (Navajas-Peréz *et al.* 2006), revealed to be effective for determining gender in *R. thyrsiflorus*. Amplification of male-specific repetitive sequence RAYSI showed the presence of 930 bp product.

Additionally, amplification with primers R730-A and R730-B (Navajas-Peréz *et al.* 2005), which amplify the repetitive RAE 730 sequence located on *Rumex* autosomes was carried out to verify template DNA quality. PCR products were obtained for all analyzed explants, showing that the DNA templates used for gender determination were of good quality.

According to Rychlewski & Zarzycki (1986) the sex ratio in *R. thyrsiflorus* seed samples originating from various wild populations was slightly female-biased (1.1-1.6). An average prevalence of female from nature might be expressed with the ratio 1:1.25. The constant predominance of female seeds might result from e.g. a higher mortality of male zygotes or embryos, but a factor most significantly influencing the sex ratio in populations of *R. thyrsiflorus* seems to be the differential survival rate (Rychlewski & Zarzycki 1986).

The results of our preliminary studies revealed the female-biased sex ratios among explants cultured *in vitro* (M:F=1:1.7). The female-biased sex ratios in case of explants showed organogenesis *in vitro* (M:F=1:2.44) may suggest a higher regeneration ability of female explants. Obtained results seems to be very interesting and future studies concerning some physiological differences (e.g. proteins related to stress responses, antioxidant enzymes, level of endogenous growth regulators), which could determine different morphogenetic reaction of male and female explants under *in vitro* conditions, are needed.

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