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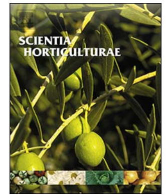

Research paper

In vitro tetraploid induction of *Malus* × *domestica* Borkh. using leaf or shoot explants

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ABSTRACT

Triploids and tetraploids of *Malus* × *domestica* Borkh. are widely used in breeding programmes because they are characterised by lush growth, larger organs and greater resistance to biotic and abiotic stress. The aim of this study was to develop an *in vitro* method of apple polyploidisation using leaf and shoot explants of six cultivars. At first, the procedure of efficient *in vitro* shoot regeneration from leaves was optimised. The leaf regeneration capacity was generally increased by preculture of donor shoots with 4.5 μM thidiazuron (TDZ) compared to a standard preculture in the presence of 4.5 μM benzyladenine (BA). Therefore, for polyploidisation, the leaf explants were collected from four-week shoot cultures that were pretreated with TDZ and the shoot explants derived from standard four-week multiplication subculture. The explants were incubated for six days in darkness on induction medium containing 2.5 μM 1-naphthaleneacetic acid (NAA) and 4.5 μM TDZ or 18 μM BA (leaves) or and 0.5 μM indole-3-butyric acid (IBA) and 4.5 μM BA (shoots) and one antimitotic agent: colchicine, trifluralin, oryzalin or amiprophos methyl (APM). Subsequently, the explants were cultured for four weeks in darkness on the medium without antimitotic agents and then subcultured on multiplication medium containing BA over a 16-h photoperiod. From leaf explants, 58, 38 and 6 tetraploids were obtained for three out of six cultivars, 'Pinova', 'Redchief' and 'Sander', respectively. For leaf explants, the highest polyploidisation efficiency, approximately 20%, was recorded for colchicine at 125 and 250 mg l^{-1}. For shoot explants, tetraploids were detected for all the cultivars with the higher tetraploid numbers – 13, 26 and 27, for Co-op 32, 'Free Redstar' and 'Redchief', respectively – and a few tetraploids were obtained for other genotypes ('Gala Must.', 'Sander' and 'Pinova'). For shoot explants, treatment with 10 mg l^{-1} APM resulted in the highest polyploidisation efficiency of 9.8%. Additionally, mixoploids were detected three times more than tetraploids when shoots were used for polyploidisation, compared to the sporadic occurrence of mixoploids when leaf explants were used.

1. Introduction

One of the important sources of variability is the process of polyploidisation. Polyploids are the genotypes that contain more than two sets of chromosomes. They are widely used in breeding programmes for crops because they result in lush growth, larger flowers and fruits, a smaller number of flowers per inflorescence, sometimes shorter and more compact shoot habit or greater resistance to biotic or abiotic stress (Chen, 2007; Dhooghe et al., 2011; Mason, 2016).

Polyploidisation has been used to breed crops for decades (Mason, 2016). It is well documented that polyploidisation leads to novel phenotypes through several mechanisms; for example, alterations in dose-regulated gene expression and rapid genetic and epigenetic changes (Parisod et al., 2010; Mason, 2016). Recently, several *in vitro* methods of mitotic chromosome doubling have been developed for plants such as *Pyrus communis* (Sun et al., 2011), *Actinidia* sp. (Wu et al., 2011), *Humulus lupulus* (Trojak-Goluch et al., 2013), *Hemerocallis* (Podwyszyńska et al., 2015), raphanobrassica (Niimi et al., 2015) and *Populus* (Xu et al., 2016).

Apple species and cultivars differ in the number of chromosome sets. The majority of the apple cultivars are diploids (2x = 34), but there are some triploids (3x = 51) and a few tetraploids (4x = 68) (Korban et al., 2009; Considine et al., 2012; Podwyszyńska et al., 2016). Triploid and tetraploid apple genotypes are generally characterized by their large fruits and often display other phenotypes that are valuable to both growers and consumers (Janick et al., 1996; Sedysheva and Gorbacheva, 2013; Podwyszyńska et al., 2016). The mentioned above authors estimated that approximately 10% of the commonly grown

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cultivars were triploids.

Considine et al. (2012) demonstrated that all apple triploids obtained naturally from sexual polyploidisation were derived from 2*n* ova fertilised with *n* pollen. These authors reported that within the F_1 population of diploid *Malus* parents, there were only 0.199% triploids and 0.05% tetraploids. Therefore, mitotic polyploidisation may allow for more tetraploids to be obtained through apple breeding. In our previous publication in which the phenotype of cultivated apple cultivars was evaluated, it was demonstrated that when diploids, triploids and tetraploids were compared, polyploidy was generally associated with an increase in the size of stomata, leaves, flowers and fruits (Podwyszyńska et al., 2016). Furthermore, despite the relatively low pollen viability observed in apple triploid and tetraploid cultivars, such genotypes formed a little viable pollen (Podwyszyńska et al., 2016) with probable some diploid pollen, as it was proved by Sedysheva and Gorbacheva (2013). That means that apple cultivars with either triploid or tetraploid genotypes can be used as pollinators for crossing with diploid genotypes in order to obtain new triploid cultivars. Neotetraploids may also become new cultivars. It was shown that apple tetraploids were characterized with enhanced productivity or fruit quality (Sedyscheva and Gorbacheva, 2013; Liu et al., 2006) and acquired increased resistance to abiotic stress as it was shown for mitotic tetraploids of apple rootstock *Malus zumi*, which revealed increased salt and drought resistances compared to its diploid counterpart (Jia, 2009). Many reports on several crop species indicate that tetraploids are more resistant to disease than their diploid progenitors due to a duplication of resistance genes and/or alteration of their molecular function as a consequence of whole genome duplication (Panchy et al., 2016). Several authors reported successful production of mitotic tetraploids of apple cultivars and rootstocks using *in vitro* methods (Bartish et al., 1999; Liu et al., 2006; Ou et al., 2008; Jia 2009). All these authors induced tetraploids from leaves. On the other hand, the shoot explants of the apple were used sporadically and did not result in tetraploids (Jia, 2009).

In mitotic polyploidisation, chromosome doubling occurs in somatic cells. In order to induce polyploids, the most commonly used antimitotic agent is colchicine (Dhooghe et al., 2011). However, due to the carcinogenic action of colchicine on the mammalian microtubuli, including in humans, there has been an increase in the use of other antimitotic agents that are considered less toxic to human health, such as oryzalin, trifluralin and amiprophos methyl (APM) (Dhooghe et al., 2009a,b; Podwyszyńska, 2012; Niimi et al., 2015). For apple polyploidisation, only colchicine and oryzalin were used (Bartish et al., 1999; Ou et al., 2008; Jia, 2009).

The overriding objective of our research is to obtain tetraploids that are characterized by increased productivity and enhanced resistance to abiotic and biotic stress such as resistance to serious apple diseases: apple scab, fire blight or powdery mildew. The aim of the presented study was to develop a complete method for *in vitro* polyploidisation of apples using both the leaf and shoot explants and various antimitotic agents, including APM and trifluralin not used before for chromosome doubling of *Malus* sp.

One of the factors that contributes to successful apple mitotic polyploidisation *in vitro* is the efficiency of direct regeneration of adventitious shoots from the explants such as leaves (Bartish et al., 1999; Ou et al., 2008). Therefore, the first step of our research on apple polyploidisation was to optimise the efficiency of *in vitro* shoot regeneration from leaves.

2. Materials and methods

2.1. Plant material and in vitro culture conditions

Six cultivars with high or moderate resistance to serious apple diseases such as apple scab and/or fire blight or powdery mildew were used for the study: ‘Free Redstar’, ‘Gala Must’, ‘Pinova’, Co-op 32, ‘Redchief’ and ‘Sander’. *In vitro* shoot cultures were established and multiplied by stimulating axillary shoot growth. Shoots were established and continuously multiplied *in vitro* at four to five week subculture periods, in 330 ml jars that each contained 40 ml of standard multiplication medium. The medium was composed of modified Murashige and Skoog (1962) macro- and microelements in which the iron source was changed for the chelate EDDHA (80 mg l^{-1}), 3% sucrose, inositol (100 mg l^{-1}), glycine (2 mg l^{-1}), thiamine (1 mg l^{-1}), nicotinic acid (1 mg l^{-1}) and pyridoxine. The multiplication medium was supplemented with 4.5 µM benzyladenine (BA), 0.3 µM gibberellic acid (GA_3) and 0.5 µM indole-3-butyric acid (IBA), adjusted to a pH of 5.6 and solidified with 6 g l^{-1} agar (Plant Propagation Labagar, Biocorp, Poland). This basic medium, with the exception of the growth regulators (PGRs), was used throughout the experiments.

Shoot cultures in all the experiments, except when stated, were maintained at 21 °C under a standard 16/8 h photoperiod and with a photosynthetic photon flux density of 30 µmol $m^{-2} s^{-1}$ (warm white fluorescent lamps).

2.2. Optimisation of shoot regeneration from leaf explants and histological analysis

Leaves were collected from *in vitro* shoot cultures. The donor shoots were precultured for four weeks on a basic multiplication medium containing thidiazuron (TDZ) or BA, each at the concentration of 4.5 µM. Other plant growth regulators (PGRs), GA_3 and IBA, were also used as in the basic multiplication medium. The third and fourth youngest leaves were taken from these four-week shoot pre-cultures and then incubated (with abaxial surface in contact with the medium) in darkness for four weeks on the induction medium, which contained the basic medium supplemented with the PGRs, TDZ (4.5 µM) or BA (18 µM) in combination with 1-naphthaleneacetic acid (NAA) (2.5 µM). Next, for adventitious shoot regeneration, the leaf explants with callus and regenerating shoots were cultured for the following four weeks on a regeneration medium that contained BA (2.25 µM), IBA (0.25 µM) and GA_3 (1.5 µM) and then on a standard multiplication medium with a 16/8 h photoperiod.

The percentage of leaves that formed callus and/or shoots, the number of shoots and the size of the callus was estimated after being cultured for four weeks on the induction medium. The following grading scale was used: no callus formation (1), callus diameter 1–2 mm (2), 3 mm (3), 4 mm (4) 5 mm and more (5). The percentage of explants that regenerated shoots and number of shoots that formed on leaf explants were also noted at the end of experiment (that is, after eight weeks of shoot regeneration, i.e. four-weeks on the induction medium + four weeks on the regeneration medium).

Histological analysis was performed to determine the type of shoot regeneration that resulted from the leaf explants depending on PGRs (TDZ and BA) used in the pre-culture and induction medium, as described above. The analysis was performed for the cultivar Co-op 32. Plant materials with developing structures were sampled three times: after two- and four-week culture on induction medium and then after another four-week culture on regeneration medium. The morphological features of regenerated structures were evaluated using a stereoscopic microscope Olympus SZX 16 with cellSens imaging software. The samples were fixed in chromic acid, acetic acid and formalin (CrAF) for 48 h, dehydrated through an alcohol series, embedded in paraffin and sectioned to 15 µm on a rotary microtome. Cut material, after staining with safranin and fast green, was closed in Canada balsam and examined by conventional light microscopy Nikon Eclipse 80i with imaging software NIS-Elements Br 4.00 for photodocumentation.

2.3. Polyploidisation using leaf explants

Based on the results of the experiment on optimization of shoot regeneration from leaves, 4.5 µM BA was used as optimal in the preculture medium for donor shoots of ‘Free Redstar’ and 4.4 µM TDZ in

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