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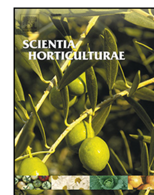

The use of TDZ for the efficient *in vitro* regeneration and organogenesis of strawberry and blueberry cultivars

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ABSTRACT

Application of *in vitro* plant biotechnology in berry crops depends on the availability of efficient regeneration protocols that are specific to the genotype background and to the correct combination of exogenous hormones (auxin and cytokinin) added to the medium. Strawberry regeneration protocols are available for different cultivars, while more limited information are available for blueberry cultivars. In this study, we show that the best regeneration efficiency for the leaves of strawberry cultivar Calypso were obtained culturing in a medium supplemented with thidiazuron (TDZ) 0.5 mg L^{-1} and 2,4-dichlorophenoxyacetic acid (2,4-D) 0.02 mg L^{-1}. The best regeneration efficiency for cultivar Sveva leaves was obtained culturing in a medium supplemented with N6-benzyladenine (BA) 3 mg L^{-1} and indole-3-butyric acid (IBA) 0.2 mg L^{-1}.

In blueberry cultivar Duke, shoot proliferation trials were carried out comparing the effects of 2-isopentenyladenine (2iP) and TDZ; furthermore, experiments of blueberry direct and indirect organogenesis were made, using TDZ as alternatives to zeatin, the most common and expensive hormone used for blueberry *in vitro* regeneration. Different regeneration responses were observed by using TDZ alone or combined with 2iP. The addition of both 0.2 mg L^{-1} or 0.5 mg L^{-1} of TDZ in the medium led to improved callus formation. The addition of 15 mg L^{-1} of 2iP in the same medium promoted blueberry stem elongation, while inhibiting callus growth. Zeatin was most efficient in direct regeneration of shoots while 0.5 mg L^{-1} TDZ induced a highest number of shoots by indirect organogenesis.

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1. Introduction

Berries, such as strawberry and blueberry, are some of the most cultivated and consumed fruit crops worldwide and are known to be a major natural source of anthocyanin, polyphenols, folates and antioxidants (Diamanti et al., 2014; Prior et al., 1998). Methods for improving high quality production combined with reduced impact on the environment are being studied, which include development of efficient *in vitro* protocols for the production of true-to-type certified vegetative material and for the application of advanced biotechnologies for the genetic improvement of berries, including the genetic transformation techniques. Plant cells, organs and tissues exhibit a remarkable ability to regenerate new organs under *in vitro* conditions (Pulianmackal et al., 2014). Various types of organs, vegetative and reproductive, have been successfully regenerated from hundreds of plant species (Bell et al., 2012; Pathi and Tuteja 2013). So far *in vitro* somatic organogenesis has been proven to be an important system for investigating mechanisms of plant organ development and also for the application of genetic engineering technology. Adventitious shoot regeneration from *in vitro* cultured explants of cultivated strawberries has been demonstrated for a variety of tissues. Leaf discs remain the most successful and widely used regeneration explant (Nehra et al., 1990; Sorvari et al., 1993; Cappelletti et al., 2015). In addition many other starting tissues have been tested such as: petioles (Focault and Letouze 1987; Rugini and Orlando 1992), stipules (Rugini and Orlando 1992), stem tissues (Graham et al., 1995) runner tissue (Liu and Sanford 1988), the peduncular base of the flower bud (Focault and Letouze 1987), mesophyll protoplast (Nyman and Wallin 1988), anther cultures (Owen and Miller 1996), roots (Rugini and Orlando 1992) and immature embryos (Wang et al., 1984). For the development of *in vitro* regeneration protocol, the role of exogenous hormones is widely regarded as being the most important factor (Azad et al., 2004; Subotic et al., 2009). Auxin and cytokinin are crucial for the regulation of organ regeneration, and the concentra-

Abbreviations: TDZ, thidiazuron; 2,4-D, 2,4-dichlorophenoxyacetic acid; BA, N6-benzyladenine; IBA, indole-3-butyric acid; 2iP, 2-isopentenyladenine; PGR, plant growth regulator.

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tion ratio between these hormones is critical to determine specific organogenesis processes from different somatic tissues (leaf, root, *etc.*) (Christianson and Warnick 1985; Su et al., 2011). The interaction between plant cytokinins and auxins are complex and roots, shoots and callus formation are highly influenced by different combinations and concentrations of plant growth regulators (PGRs). The cytokinin and auxin combination of Benzyl adenine (BA)/Indol Butiric Acid (IBA) is most commonly used for strawberry shoot multiplication. For some genotypes, this PGR combination is also used to induce good regeneration of genetically transformed strawberry plants (Koskela et al., 2012; Mouhu et al., 2013; Zhang et al., 2014).

Generally, Thidiazuron (TDZ), a cytokinin-like compound widely used to promote shoot proliferation and regeneration in strawberry (Barceló et al., 1998; Oosumi et al., 2006; Landi and Mezzetti 2006), can replace the use of BA. TDZ produces different regeneration responses depending on the plant genotype and the starting explant used (Passey et al., 2003). TDZ is a phenylurea that induces a high morphogenesis activity in strawberry leaf tissues (Passey et al., 2003) and it's involved in the synthesis of auxin by increasing the levels of indole-3-acetic acid (IAA) and its precursor tryptophan (Murthy et al., 1998). During the last years, TDZ has been successfully tested in combination with the auxin 3-benzo[b]selenienyl acetic acid (BSAA), mostly for the induction of somatic embryogenesis in several woody crops (Lamproye et al., 1990), but also to induce organogenesis in different *Fragaria* × *ananassa* genotypes (Landi and Mezzetti 2006). Recently, BSAA has been removed from the market, and therefore new studies are needed to identify new PGR combinations and efficient organogenetic protocols for regeneration of plants from strawberry leaves. It has been reported that TDZ alone, or in combination with 2,4-dichloro-phenoxy-acetic acid (2,4-D) or IBA, is effective for shoot regeneration in strawberry tissue culture (Passey et al., 2003; Debnath, 2005). TDZ combined with IBA were also effective for shoot multiplication of wild strawberries (*F. viridis* Duch.) (Ghasemi et al., 2015). 2,4-D is an auxin growth regulator, also frequently used in strawberry regeneration experiments, and Folta et al. (2006) obtained better regeneration results in some cases by a more than 10-fold reduction in its concentration in the medium, compared to previous studies (Nehra et al., 1990).

Highbush blueberry is a soft fruit crop of large interest due to the high value of their edible fruits, and excellent source of health-promoting nutrients. The interest in large-scale production of *Vaccinium* species and their genetic improvement is growing constantly, due to the increasing commercial interest of these small fruits (Ostrolucká et al., 2007). Micropropagation is one of the best methods for the rapid propagation of elite plants and by the end of 1980s more than one million highbush blueberry plants were propagated annually worldwide (Litwińczuk, 2013). Thus, the development of efficient *in vitro* propagation protocols for blueberry varieties has become more important, particularly because of the difficulties encountered during *in vivo* rooting of some cultivars, and problems in obtaining plants genetically identical to their mother plants, due to their genetic heterozygosity (Debnath 2007; Fira et al., 2008; Ostrolucká et al., 2007).

Knowledge and advances in micropropagation techniques are very important to optimize protocols of proliferation and regeneration of blueberry tissues for cryopreservation (Shibli et al., 1999), *in vitro* selection of new genotypes (Finn et al., 1991), interspecific and intersectional hybrids (Zeldin and McCown, 1997) and genetic transformation (Song and Sink, 2004).

In blueberry commercial micropropagation, Zeatin (Reed and Abdelnour-Esquivel 1991; Ružić et al., 2012) and BA (Tirone et al., 2011) are the most important cytokinins used depending on the varieties. However, due to the high cost of Zeatin, studies are needed to identify new PGR combinations to substitute or reduce the expensive use of zeatin for both commercial micropropagation and *in vitro* regeneration of blueberry varieties (Reed and Abdelnour-Esquivel, 1991; Ružić et al., 2012).

Table 1
Media used for in vitro regeneration trials from strawberry leaves. Plant growth regulator values are reported in milligram per litre.

Media code	Growth regulator			
	TDZ	BA	2,4-D	IBA
TDZ-a	1.0		0.2	
TDZ-b	0.5		0.02	
BA-a		3		0.2
BA-b		1.5		0.1

The general aim of this research was to study the efficiency of TDZ (a cytokinin-like PGR), alone or combined with other PGRs, in inducing leaf tissue shoot regeneration in strawberry, and shoot multiplication and leaf tissue regeneration in blueberry. In strawberry, TDZ/2,4-D and BA/IBA combinations, at different concentrations of the different PGRs, were compared for inducing the highest rate of shoot regeneration from leaf tissues of Calypso, an everbearing variety, and Sveva, a short day variety. In parallel, the development of efficient methods for the induction of *in vitro* shoot multiplication and leaf tissue regeneration efficiency in *Vaccinium corymbosum* L. var. Duke were investigated. In particular, the ability of TDZ alone or in combination with 2iP, to induce high regeneration efficiency, callus formation and inhibition of the elongation, were studied.

2. Material and methods

2.1. Strawberry establishment in *in vitro* culture

Two octoploid cultivars of *Fragaria* × *ananassa* were used for this study: Calypso, an ever-bearing cultivar and Sveva, a short day cultivar. The two cultivars, grown in the experimental farm of the Marche Polytechnic University, were prepared for *in vitro* conditions by sterilizing apical buds collected from runners, and treating them with a 2% (v/v) chloride-active solution for 20 min. After rinsing three times with sterile distilled water, the developed shoots were transferred to tubes that contained the basal medium of salts and vitamins as described by Murashige and Skoog (1962) (MS medium; 4,40 g L^{-1}, Duchefa preparation), supplemented with 3% sucrose (w/v), 0.25 mg L^{-1} BA and 7.5 g L^{-1} Agar (Plant agar, Duchefa). The pH value of the media was adjusted to 5.8–5.7 before autoclaving. Once the shoot proliferation was stabilized in the MS medium, some of stock plants were transferred to the elongation medium (MS without plant growth regulator and with other miscellaneous previously described) in order to have in 4 weeks new expanded leaves useful for regeneration trials. Plants subjected to proliferation and elongation steps were kept in a growth chamber at 24 ± 2 °C under a 16 h photoperiod at 250 μmols m^{-2} s^{-1}, and sub-cultured regularly at 4-week intervals.

2.2. Strawberry *in vitro* regeneration experiments

Strawberry regeneration experiments were performed using young expanded leaves detached from 4-weeks-old *in vitro* elongated plants obtained after a minimum of 4–5 subcultures from initial explants, following the propagation strawberry protocol described above (Fig. 1a). The leaf laminas were cut transversally along the leaf mid vein and cultured with the abaxial surface in contact with the MS regeneration medium, supplemented with 3% sucrose (w/w), 7.5 g L^{-1} plant agar (Duchefa) and different combinations of the PGRs TDZ, BA, 2,4-D and IBA (Table 1). The pH value of the media was adjusted to 5.8–5.7 before autoclaving. The explants were cultured in 9 cm Petri dishes kept in the growth chamber at

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