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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.

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 (Ostroluckà 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; Ostroluckà 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

Fig. 1. (a) Four-week-old *in vitro* elongated strawberry plant placed on elongation medium. (b) Strawberry leaf explants cut transversally after 2 weeks of culture in dark condition.

25 °C, in the dark for the first two weeks and then sub-cultured on fresh media every two weeks, for three subsequent subcultures, placed under a photoperiod of 16 h at 250 μmol m^{-2} s^{-1} (Fig. 1b).

The regeneration response was monitored for the following parameters: percentage of leaves producing callus and/or regenerating adventitious shoots and the number of shoots per regenerating leaf explant.

2.3. *Blueberry establishment in* in vitro *culture*

In vivo shoots of highbush blueberry, cv. Duke, were taken from plants grown in the experimental farm of the Marche Polytechnic University. For *in vitro* initiation, uniform twigs were selected and leaves were removed and cleaned with detergent and rinsed under tap water for 30 min. Nodal segments (2–3 cm) were sterilized for 20 min with commercial bleach (3.5% commercial bleach with a concentration <5% of sodium hypochlorite), adding a few drops of Tween and finally washed 3 times × 15 min, in sterile distilled water, under a laminar flow cabinet to ensure sterile condition.

Sterilized shoots were proliferated and maintained on media consisting of the WPM (Lloyd and McCown, 1980) salts and vitamins combination, supplemented with 30 g L^{-1} sucrose, zeatin 2 mg L^{-1} and plant agar 7.5 g L^{-1}. The pH value of the media was adjusted to 4.9–4.8 before autoclaving. The explants were kept in the growth chamber at 25 °C, placed under a photoperiod of 16 h at 250 μmol m^{-2} s^{-1}.

2.4. *Blueberry* in vitro *proliferation and regeneration experiments*

For blueberry shoots *in vitro* proliferation, the WPM (McCown Woody Plant Medium) (Lloyd and McCown, 1980), salts and vitamins combination, as described by Gajdosova and Laimer (2009) was used, supplemented with 30 g L^{-1} sucrose, and different concentrations and combinations of TDZ, Zeatin and 2iP (Table 2). The pH of all media was adjusted to a final pH of 4.9–4.8 with HCL or KOH before autoclaving. The explants were transferred to fresh media every two weeks for three subsequent subcultures, kept in the growth chamber at 25 °C and placed under a photoperiod of 16 h at 250 μmol m^{-2} s^{-1}. The shoot proliferation rate induced by the different PGR treatments was monitored by recording the number of shoots per explant combined with other variables; shoot length, callus width and number of leaves.

In parallel, different regeneration trials were carried out to investigate regeneration capability of blueberry through direct and indirect organogenesis. Blueberry young leaves, from *in vitro* shoots proliferated on WPM1, cut perpendicularly to the central vein were used as starting explants for direct organogenesis experiments. Direct organogenesis was investigated by placing the blueberry leaves on different regeneration media, containing the WPM salts and vitamins combination, supplemented with 30 g L^{-1} sucrose and the addition of different PGRs as shown in Table 2. The induction of indirect organogenesis was investigated applying the meristematic bulk regeneration method used by Mezzetti et al., 2002; with some modifications. Blueberry meristematic bulks (MBs) were initiated by placing blueberry *in vitro* shoots (previously proliferated on WPM1) on WPM4 for six weeks, sub-culturing the explants on fresh media every two weeks at a constant concentration of TDZ 0.5 mg L^{-1}. Callus slices belonging from the blueberry MBs so obtained, were placed on WPM2, WPM3, WPM4 and WPM6 to investigate the ability of the different WPM media hormonal composition to induce indirect organogenesis (Table 2). The percentage of regenerating explants and the number of shoots regenerated per leaf/or callus were recorded for both direct and indirect organogenesis experiments.

3. Statistical design and analysis

All the regeneration and proliferation experiments have been designed with a complete randomization of the PGR combinations with 3 and 5 replicates, for strawberry and blueberry respectively, for each treatment. Each replicate consisted of 10 explants placed in jars (for proliferation) or Petri dishes (for leaf regeneration). The results were scored after 42 days, and they were analyzed as the means of the observations from three independent experiments.

Table 2
Media used for blueberry shoot in vitro regeneration trials and for blueberry leaf and callus in vitro direct and indirect organogenesis experiments. Plant growth regulator values are reported in milligram per litre.

Media code	Growth regulator				
	TDZ	Zeatin	2iP	TDZ	Zeatin
	Proliferation			Direct and indirect organogenesis	
WPM1 (no additions)					
WPM2	0.2			0.1	
WPM3	0.2		15	0.2	
WPM4	0.5			0.5	
WPM5 (Used only for shoot proliferation)	0.5		15		
WPM6		2			2

Data were analyzed using ANOVA and significant differences were calculated using the LSD test.

4. Results and discussion

The aim of this study was to find the best regeneration media for the leaf regeneration of two strawberry varieties, Calypso and Sveva. Strawberry has been largely regenerated starting from several different explants, but regeneration capability has been demonstrated to be extremely correlated to genotype background, so studies and experimental tests must be done in order to ensure good regeneration efficiency for each cultivar of commercial interest. The use of TDZ (a cytokinin-like PGR) combined with 2,4-D was compared to the combination BA/IBA, and all the PGRs were used at different concentrations in order to find the best hormone balance able to induce leaf organogenesis. In addition this study was designed to compare the ability of TDZ and 2iP to induce shoot proliferation, regeneration and callus formation in blueberry, cv. Duke, in order to find a possible alternative hormone to replace the expensive application of zeatin for blueberry *in vitro* propagation.

4.1. *Effect of different PGRs on strawberry* in vitro *regeneration*

The analysis of variance showed significant effects on callus formation and shoot regeneration capacity by placing leaves of the two strawberry genotypes on media containing different PGRs (Table 3). The explants were monitored periodically and the data were scored after 42 days of culture, when the tissues seemed to have reached the maximum regeneration level. For Sveva, explants cultured on BA-a medium showed the highest percentage of callus formation and of regenerating leaves (98% of leaves with calli and 80% of leaves regenerating shoots) associated with the highest number of regenerated shoots per explant (10.3 shoots per leaf). A high callus formation (90% of leaves with calli), associated with the final high regeneration of shoots (7.0 shoots per leaf), was observed also by applying the PGRs combinations of TDZ-a medium. However, the percentage of leaves that regenerated shoots was lower compared to the results obtained with BA-a medium (67% of leaves regenerating shoots). All the other PGRs combinations induced lower callus formation and shoot regeneration. On Calypso explants TDZ, applied at both concentrations, induced high callus formation (100% of leaves with calli), but the best regeneration efficiency (95% of leaves regenerating shoots with 11.7 shoots per leaf) was induced using TDZ-b medium. The use of BA as exogenous cytokinin induced less callus formation on Calypso leaves, with 90% of explants cultured on BA-a and only 60% on BA-b. This reduced callus formation was also associated with the lowest shoot regeneration efficiency at both BA concentrations used (Table 3).

Results demonstrated that genotype effect is the main factor affecting the regeneration response to the different PGR treatments. In particular, Calypso, the everbearing cultivar, is known as a high-regenerative genotype by traditional micropropagation and organogenesis experiments (Passey et al., 2003; Landi et al., 2009). When this plant was cultured in media supplemented with TDZ combined with 2,4-D, there was high callus formation in both doses of TDZ (0.5–1 mg L^{-1}) (Fig. 2a and b); this effect was previously investigated by several authors (Passey et al., 2003; Folta et al., 2006; Landi and Mezzetti 2006). Interestingly, we noticed that the percentage of regenerating explants and numbers of regenerated shoots were higher using 0.5 mg L^{-1} of TDZ, although a high callus formation was observed in both regeneration media used (TDZ-a and TDZ-b). Therefore, Calypso showed good regeneration capability also using lower concentration of both TDZ and 2,4- D, compared to other protocols (Passey et al., 2003; Hanhineva et al., 2005; Qin et al., 2005a,b), confirming the approach already proposed by Folta et al. (2006), which is also useful to avoid risks of somaclonal variation. In order to understand the best regeneration medium for Sveva and the best hormone ratio and concentration, the effect of TDZ combined with 2,4-D was compared with the combination of BA with IBA. As reported in literature by Oosumi et al. (2006), BA also has a specific role in strawberry regeneration. Although callus formation was comparable in both PGRs combinations applied, a higher percentage of regenerated explants and number of shoots per leaf were obtained on the media supplemented with BA. This confirmed its greatest efficiency in inducing leaf tissue regeneration. In particular, using the medium BA-a we obtained more efficient regeneration activity of Sveva leaves and we adopted this hormone concentration for the regeneration trials (Fig. 2c). On the contrary, Sveva calli obtained culturing leaves on TDZ-a and -b were notably hard and yellowish compared to those obtained using BA-a (Fig. 2d), this could have been the cause of the low regeneration efficiency observed.

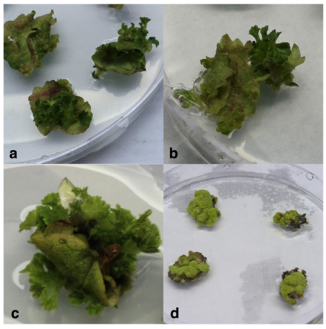

Fig. 2. Calypso regenerated shoots via organogenesis after 4 weeks of leaf culture in (a) TDZ-a medium and in (b) TDZ-b medium. (c) High regeneration capability observed in Sveva leaves cultured in BA-a medium. (d) Hard and yellowish callus obtained culturing Sveva leaves in TDZ-a medium.

4.2. *Blueberry* in vitro *proliferation*

The use of TDZ as cytokinin for plant *in vitro* shoot proliferation was investigated on blueberry cv Duke alone or combined with

Table 3
Effects of PGR combinations on the number of shoots from the regenerating leaves of cultivars Sveva and Calypso after 42 days of culture.

	Sveva[a]			Calypso[a]		
Media	Leaves with calli (%)	Leaves with shoots (%)	n° shoots/leaves	Leaves with calli (%)	Leaves with shoots (%)	n° shoots/leaves
TDZ-a	90	67	7 ± 1^{b}	100	78	6.7 ± 1.2^{b}
TDZ-b	80	70	5 ± 1^{c}	100	95	11.7 ± 1.5^{a}
BA-a	98	80	10.3 ± 0.6^{a}	90	70	5.3 ± 1.5^{c}
BA-b	67	55	2.3 ± 0.6^{d}	60	47	2 ± 1^{d}
MS0	0	0	0.3 ± 0.6^{e}	0	0	0 ± 0^{d}

Means in columns with different letter are significantly different according to LSD test ($P < 0.05$).
[a] Data are means ± s.e.m.

Table 4
Number of shoots, callus size, shoot length and number of leaves detected after 6 weeks of cultivation of Blueberry (Duke).

Media	n° shoots/plantlet[a]	shoots length[a]	callus width[a]	n° leaves/plantlet[a]
WPM1	0.3 ± 0.4^{c}	27.2 ± 14.4^{a}	2.5 ± 1.41^{dc}	9.8 ± 5.12^{b}
WPM2	1.1 ± 0.9^{c}	7.8 ± 2.26^{a}	9.9 ± 2.67^{a}	6.6 ± 5.6^{c}
WPM3	0.7 ± 0.6^{b}	16.7 ± 11.32^{b}	1.9 ± 0.66^{d}	11.5 ± 7.55^{b}
WPM4	0.6 ± 0.9^{b}	8.2 ± 2.95^{b}	9.1 ± 3.57^{b}	5.7 ± 5.15^{c}
WPM5	0.6 ± 0.8^{b}	12.7 ± 4.78^{b}	3.2 ± 2.33^{d}	8.9 ± 6.3^{b}
WPM6	3.5 ± 2.1^{a}	29.1 ± 8^{b}	6.6 ± 1.98^{c}	36.5 ± 24.11^{a}

Means in columns with different letter are significantly different according to LSD test ($P < 0.05$).
[a] Data are means ± s.e.m.

2iP (normally used during the proliferation phase) to reduce and replace zeatin (more commonly used in blueberry *in vitro* propagation medium).

TDZ, as unique PGR added to the medium, induced especially to a high callus formation at the bases of shoots, at both concentrations tested (WPM2 and WPM4) (Fig. 3a and b). Placing blueberry explants on medium containing 2iP (WPM3 and WPM5) we found that this compound promotes elongation of the stems and inhibits callus formation if combined with TDZ (Fig. 3c and d), demonstrating that this PGR combination could be a possible solution for blueberry shoot elongation of the more recalcitrant blueberry cultivars. Shoots placed on medium supplemented with 2 mg L^{-1} zeatin (WPM6), showed balanced morphogenesis (Fig. 3e), the best proliferation rate (3.5 shoots per plantlet) and the highest shoot elongation (Table 4) compared to the explants placed on all other treatments (Fig. 3f). This confirmed the efficiency of this cytokinin for the *in vitro* proliferation of more common blueberry varieties (Reed and Abdelnour-Esquivel 1991; Meiners et al., 2007). Therefore, this study does not indicate that TDZ can be used as a replacement of zeatin for blueberry proliferation.

4.3. *Blueberry* in vitro *direct and indirect organogenesis*

In this study experiments of direct and indirect blueberry leaf and shoot organogenesis were also carried out; in particular, the efficacy of TDZ in shoot regeneration induction was investigated as alternative to zeatin. Direct leaf regeneration was statistically higher with zeatin (7.6 shoots per leaf) than with TDZ (3.6 shoots per leaf) (Table 5). In the regeneration trials on blueberry leaves, the efficacy of zeatin for the induction of leaf organogenesis (Table 5; Fig. 4a) confirmed the results of other authors (Reed and Abdelnour-Esquivel 1991; Cao et al., 2002; Meiners et al., 2007), although in this study lower concentrations were used compared to previous studies (Rowland and Ogden 1992; Cao et al., 2002; Song and Sink 2004). On the contrary, the use of TDZ (WPM4) was more efficient in inducing indirect organogenesis from callus lines selected with the meristematic bulk approach (Mezzetti et al., 2002); in particular, a higher regeneration efficiency was observed on blueberry tissues placed on WPM4 (60% of calli regenerating shoots and 6.66 shoot per callus) as compared to that placed on WPM6 containing zeatin (30% of calli regenerating shoots and 3.5 shoots per callus) (Table 5; Fig. 4b). So, the efficiency of TDZ in inducing indirect organogenesis observed in strawberry (Landi and Mezzetti 2006; Cappelletti et al., 2015) was also observed in blueberry.

Therefore, TDZ represents a good solution for the regeneration of both strawberry and blueberry cultivars, even if specific protocols have to be developed for each genotype tested. Although zeatin represents one of the best PGRs for inducing blueberry proliferation, TDZ combined with 2iP can offer a solution for shoot proliferation and leaf tissue regeneration in the more recalcitrant blueberry cultivars. The study conducted on blueberry shows for the first time the efficacy of TDZ for the induction of indirect organogenesis, compared to the use of zeatin, applying the meristematic bulk regeneration method. The *in vitro* regeneration protocols developed in this study could be useful for several applications, such as the production of certified vegetative material and for biotechnological purposes.

5. Conclusions

The application of genetic modification technology in strawberry is related to the efficiency of the regeneration protocol. It is likely that in strawberry, the genotypes with the highest regeneration efficiency will be more useful for obtaining stable genetically modified plants, as was demonstrated with the most efficient genotypes (Mezzetti et al., 2004). For this reason, genotype effects are always important and regeneration trials have to be carried out every time a new genotype is subjected to biotechnological approaches. Hormone ratios are crucial for determining cell developmental fate during *in vitro* organogenesis and different growth regulators should always be taken into account in order to obtain best results.

Positive results in terms of strawberry regeneration efficiency were obtained culturing Calypso cultivar in a medium supplemented with TDZ 0.5 mg L^{-1} and 2,4-D 0.02 mg L^{-1}. Best regeneration efficiency was obtained culturing Sveva leaves in a medium supplemented with BA 3 mg L^{-1} and IBA 0.2 mg L^{-1}.

Zeatin remains the best PGR for inducing blueberry proliferation and shoot elongation. TDZ had the negative effect of inducing too much callus proliferation and low shoot proliferation, but if combined with 2iP can offer a solution for shoot elongation in the more recalcitrant blueberry cultivars. Similar responses of the two growth regulators were observed when used for inducing leaf tissue direct organogenesis. The effect of TDZ in inducing callus proliferation on blueberry was useful for the induction of indirect organogenesis. This is the first time that the meristematic bulk regeneration method was utilized in blueberry and in this case, TDZ resulted in more efficient shoot regeneration compared to zeatin. Therefore, both regeneration approaches can be used for the development of new protocols for blueberry genetic transformation protocols.

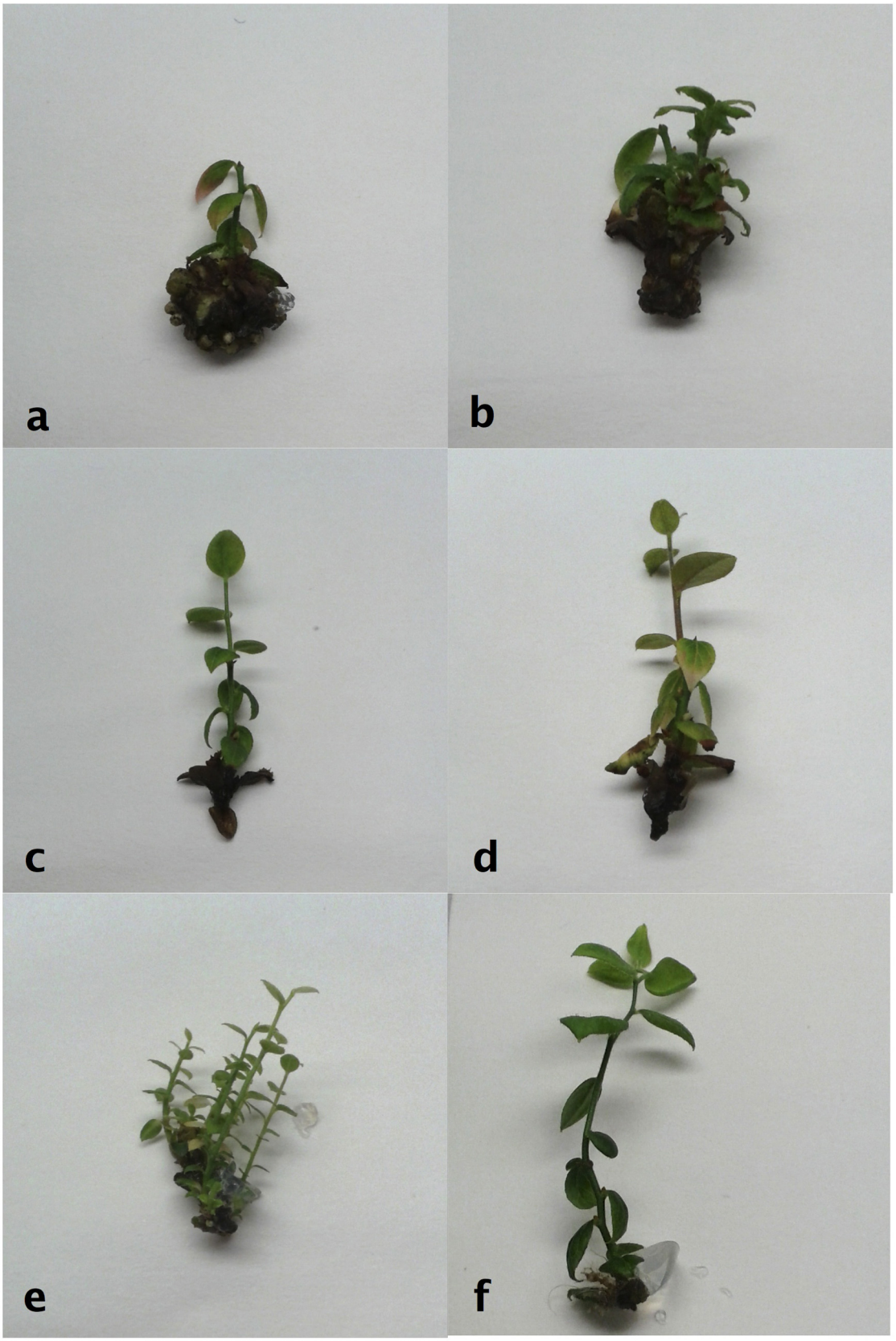

Fig. 3. (a) High callus formation on blueberry shoots regenerated on WPM2 and on (b) WPM4. Elongation and callus growth inhibition of shoots placed on (c) WPM3 and on (d) WPM5. (e) Blueberry shoots proliferation on WPM6 medium. (f) Blueberry shoots placed on WPM1.

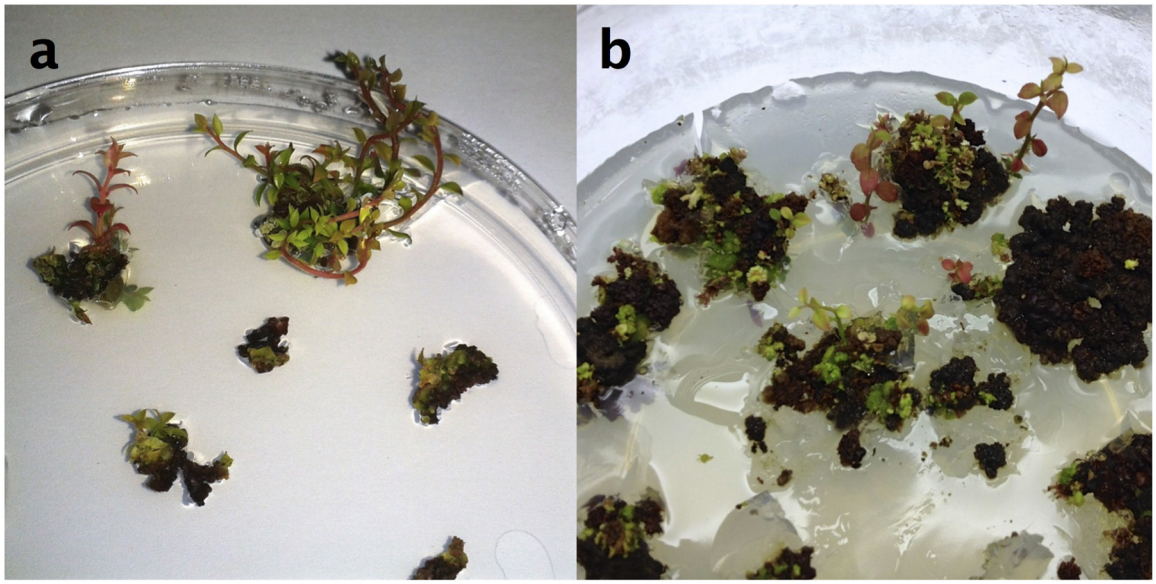

Fig. 4. (a) Direct organogenesis from blueberry leaf tissue placed on WPM6. (b) Indirect organogenesis from blueberry meristematic bulk on WPM4.

Table 5
Number of shoots regenerated through direct organogenesis and shoots produced through indirect organogenesis of Blueberry (Duke) after 42 days of culture.

	Direct Organogenesis[a]		Indirect Organogenesis[a]	
Media	Leaves with shoots (%)	n° shoots/leaves	Calli with shoots (%)	n° shoots/callus
WPM1	0	0 ± 0^{c}	0	0 ± 0^{d}
WPM2	30	1.3 ± 1.15^{c}	30	3.03 ± 0.15^{c}
WPM3	50	3.6 ± 0.57^{b}	51	4.4 ± 0.52^{b}
WPM4	55	3.6 ± 0.57^{b}	60	6.66 ± 0.76^{a}
WPM 6	70	7.6 ± 1.15^{a}	30	3.5 ± 0.5^{c}

Means in columns with different letter are significantly different according to LSD test ($P < 0.05$).
[a] Data are means ± s.e.m.

Author contribution

Roberto Cappelletti: carried out the regeneration trials, elaborated the statistical analysis and drafted the manuscript. Silvia Sabbadini: contributed to the development of the experimental design of the regeneration trials, of statistical analyses and manuscript preparation. Bruno Mezzetti: conceived the study, participated in its design and coordination and contributed to manuscript preparation.

Conflict of interest

The authors declare that they have no conflict of interest.

Key message

In vitro organogenesis of different *Fragraria* × *ananassa* cultivars and Vaccinium corymbosum (blueberry) cv. Duke using thidiazuron as the main cytokinin source.

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