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BIOCHEMICAL RESPONSES IN *AGROBACTERIUM*-INFECTED OILSEED RAPE EXPLANTS DURING EARLY STAGES OF REGENERATION IN THE PRESENCE OF DITHIOTHREITOL

Dominika Dronzeková[1], Milan Karas[2], Eva Boszoradová[3], Iwona Żur[4], Jana Moravčíková[1]*

Address(es): Assoc. Prof. Ing. Jana Moravčíková, PhD
[1] Institute of Biology and Biotechnology, Faculty of Natural Sciences, University of SS. Cyril and Methodius in Trnava, Nám. J. Herdu 2, 917 01 Trnava, Slovak Republic.
[2] Department of Agricultural and Food Sciences, DISTAL Alma Mater Studiorum - University of Bologna Viale Fanin 42, 40127 Bologna Italy.
[3] Institute of Plant Genetics and Biotechnology, Plant Science and Biodiversity Center Slovak Academy of Sciences, Akademická 2, P.O. Box 39A, 950 07 Nitra, Slovak Republic.
[4] The F. Górski Institute of Plant Physiology, Polish Academy of Sciences, Niezapominajek 21, 30-239, Krakow, Poland.

*Corresponding author: jana.moravcikova@ucm.sk

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ABSTRACT

We investigated the impact of exogenous dithiothreitol on the activities of enzymatic antioxidants ascorbate peroxidase and catalase, and the levels of non-enzymatic antioxidants and osmoprotectant proline, and total soluble sugars in *Agrobacterium*-infected and non-infected (control) explants of oilseed rape (*Brassica napus* L.) after the 3rd and 17th day of *in vitro* culture. This investigation was conducted with or without dithiothreitol at a concentration of 1 mg/L. Dithiothreitol is a sulphur-containing compound that act as a reducing agent, impacting the redox environment within plant cells. Explants were obtained from six-day-old hypocotyls of the cultivars Ability and Lancia, and infected with *Agrobacterium tumefaciens* GV3101 carrying the binary vector. The application of dithiothreitol into co-cultivation and regeneration media led to a significant decrease in the activities of ascorbate peroxidase and catalase in both infected and non-infected explants of both cultivars. In contrast, explants regenerated without dithiothreitol exhibited a significant increase in ascorbate peroxidase activity and a decrease in catalase activity, although the decrease in catalase activity was not as pronounced. The proline content increased significantly in all analysed explants, with the highest increase observed in explants regenerated in the presence of dithiothreitol. This suggests that dithiothreitol might indirectly contribute to the reduced formation of reactive oxygen species, which is generally negatively correlated with transformation efficiency.

Keywords: *Agrobacterium transformation*, ascorbate peroxidase, catalase, dithiothreitol, *in vitro* regeneration, oilseed rape, proline, total soluble sugars

INTRODUCTION

Oilseed rape (*Brassica napus* L.) is one of the most important oilseed crops in the world. Even though oilseed rape has been a significant target for breeding efforts aimed at altering the composition of fatty acids for many years, the adoption of modern biotechnological approaches still faces challenges, primarily due to genotype-dependent variations in *in vitro* regeneration efficiency (**Park *et al.*, 2012; Farooq *et al.*, 2019**). One of the obstacles hindering the successful application of transformation approaches is the response of oilseed rape tissue to *in vitro* regeneration. The composition of the medium, the cultivation conditions, the explant source, and the *Agrobacterium* transformation process could induce an elevated production of reactive oxygen species (ROS) in plant cells. Plants generate ROS as chemical by-products during the incomplete breakdown of oxygen metabolism (**Bidabadi and Jain, 2020**). ROS are pivotal in various physiological processes within plant cells (**Mansoor *et al.*, 2022**). Nevertheless, an imbalance in ROS levels can result in oxidative stress and cell death (**Choudhury *et al.*, 2016**). In the context of *in vitro* culture, elevated ROS levels are associated with tissue browning and necrosis and thus poor transformation and regeneration efficiencies (**Dan, 2008; Das and Roychoudhury, 2014; Li *et al.*, 2017**). Plants have evolved an antioxidant system comprising both enzymatic and non-enzymatic antioxidants, enabling them to effectively regulate ROS and maintain ROS homeostasis (**Hasanuzzaman *et al.*, 2020**). Several studies pointed out that exogenous antioxidants such as dithiothreitol, citric acid or lipoic acid can reduce tissue browning and improve organogenesis (**Dan, 2008; Dutta Gupta, 2010; Li *et al.*, 2017, Paes de Melo et al., 2020**). DTT is a sulphur-containing compound with free sulfhydryl groups or thiol bonds. Its main role is tied to its capacity as a reducing agent, impacting the redox environment within plant cells. DTT aids in alleviating oxidative stress by sustaining a reduced cellular environment, preserving enzyme functionality, and serving as a reducing agent for metal ions.

In this work, we aimed to study biochemical responses of oilseed rape (cultivars Ability and Lancia) during early stages of regeneration on the media supplemented with DTT. Both cultivars, Ability and Lancia, exhibited shoot regeneration efficiencies of 10% and 8.3%, respectively (**Al Ramadan *et al.*, 2021**). *Agrobacterium*-infected and non-infected (control) explants were assessed at two time points during *in vitro* regeneration: i) on the 3rd day and ii) on the 17th day. The analysis included measuring the activities of enzymatic antioxidants, such as ascorbate peroxidase and catalase, as well as assessing the contents of proline, and total soluble sugars. Ascorbate peroxidase and catalase are H_2O_2 removal enzymes (**Dumanović *et al.*, 2021**) The APX enzyme is a component of the ascorbate peroxidase cycle, capable of eliminating a relatively low level of H_2O_2 in the presence of ascorbic acid, while the CAT enzyme requires a relatively high level of H_2O_2 for its activity. Their elevated levels indicate ROS imbalance in plant cells. Proline functions as an osmoprotectant and can serve as a scavenger for ROS produced during the tissue culture process. Overproduction of proline in plant cells contributes to maintaining cellular homeostasis and mitigating oxidative damage (**Kavi Kishor *et al.*, 2015**; **Ghosh *et al.*, 2021**). Soluble sugars primarily serve as metabolic resources and structural constituents of cells, playing a pivotal role as osmoprotectants in plants. They perform diverse functions to assist plants in coping with osmotic stress (**Rosa *et al.*, 2009; Afzal *et al.*, 2021**).

MATERIAL AND METHODS

Plant material

Seeds of the oilseed rape (*Brassica napus* L.) cultivars Ability and Lancia were acquired from Norddeutsche Pflanzenzucht, Hohenlieth-Hof, Germany. The surface-sterilized seeds (100 seeds/cultivar) were germinated on the medium described by **Boszoradová *et al.* (2011)** in the dark at 23°C for 6 days.

Agrobacterium tumefaciens infection and culture conditions

The 6 days-old hypocotyls were cut into 0.5–1 cm long segments and co-cultivated with *Agrobacterium tumefaciens* GV3101 carrying the binary vector pZM4 (**Zimová *et al.*, 2019**) as was described by **Boszoradová *et al.* (2011).** Infected and non-infected (control) segments were regenerated in the liquid regeneration medium [Gamborg B5 medium, 2 % (w/v) sucrose, 250 mg/L NH_4NO_3, 750 mg/L

$CaCl_2 \cdot 2H_2O$, 250 mg/L xylose, 5 mg/L $AgNO_3$, 1 mg/L 2.4 D, 0.1 mg/L IAA, pH 5.8] supplemented with i) 1 mg/L dithiothreitol (DTT) and ii) without of DTT. Following 3 days, hypocotyl segments were transferred on solid regeneration medium [Gamborg B5 medium, 2 % (w/v) sucrose, 250 mg/L NH_4NO_3, 750 mg/L $CaCl_2 \cdot 2H_2O$, 250 mg/L xylose, 5 mg/L $AgNO_3$,1 mg/L 2.4 D, 0.1 mg/L IAA, 0.6% agar, pH 5.8] supplemented with i) 1 mg/L DTT and ii) without of DTT. Explants (162 segments/cultivar; 54 segments/replication/analyses) were cultivated at 23 °C and 16h/8h light/dark photoperiod under 10.85 µmol $m^{-2}.s^{-1}$ light intensity. Samples for analysis were collected immediately after cutting the segments, on the third day, and on the 17th day of regeneration.

Biochemical analyses

The activities of the antioxidant enzymes ascorbate peroxidase (APX) and catalase (CAT) were determined using the method described by **Kováčik *et al.*, (2009).** The enzyme activities were expressed as mmol.min^{-1}.mg^{-1} of fresh weight. Proline was quantified following the procedure outlined by **Paquin and Lechasseur, (1979)**, involving a reaction with ninhydrin, and the results were expressed in milligrams per gram of fresh weight. Total soluble sugar content was determined using the anthrone-sulfuric acid method as described by **Roe (1995)**. The content was expressed in milligrams per milligram of fresh weight.

Statistical analysis

The data are the means of three replications. All the data were examined using a two-way analysis of variance (ANOVA) followed by a post hoc comparison with the use of Duncan's multiple range test ($P \leq 0.05$). All the statistical analyses were performed using STATISTICA version 12 (Stat Soft Inc., Tulsa, OK, USA).

RESULTS AND DISCUSSION

Regeneration of plant cells in *in vitro* conditions can induce various types of stress (**Dan, 2008**). It is widely acknowledged that plant tissue injuries caused by explant preparation, the composition of the culture media or the culture conditions themselves can trigger elevated ROS production in plant cells. The impact of ROS damage relies on the scavenging ability of both enzymatic and nonenzymatic antioxidants (**Hasanuzzaman *et al.*, 2020**). Several studies reported that exogenous antioxidants such as DTT, can help to overcome poor regeneration efficiency of transformed cells (**Das *et al.*, 2002**; **Li *et al.*, 2017**). The primary function of DTT is associated with its role as a reducing agent, influencing the redox environment within plant cells. In our study, we investigated the impact of DTT on the levels of antioxidant enzymes (APX and CAT) (Figure 1) and nonenzymatic antioxidant and osmoprotectant proline (Figure 2A, 2B), and on the content of total soluble sugars (Figure 2C, 2D) in *Agrobacterium*-infected and non-infected (control) explants of two oilseed rape cultivars Ability and Lancia. Both cultivars demonstrate low *in vitro* regeneration efficiency, with Ability at 10% and Lancia at 8.3%. (**Al Ramadan *et al.*, 2021**). The concentration of DTT (1 mg/L) was selected based on our previous experiments (**Dronzeková *et al.*, 2023**). Hypocotyls explants, derived from 6-days old seedlings, were co-cultivated with *Agrobacterium* inoculum, and regenerated on the media supplemented with i) DTT and ii) without DTT (Figure 3). Additionally, regeneration media were enriched with $AgNO_3$ (5 mg/L) that is commonly used in tissue culture of oilseed rape (**Farooq *et al.*, 2019; Jiang *et al.*, 2020; Al Ramadan *et al.*, 2021).** Samples were collected from hypocotyl segments i) after their cutting into segments, ii) on the third day of the regeneration in the liquid medium and iii) after 14 days of regeneration on the solid regeneration medium. Non-infected hypocotyl segments were used as a control.

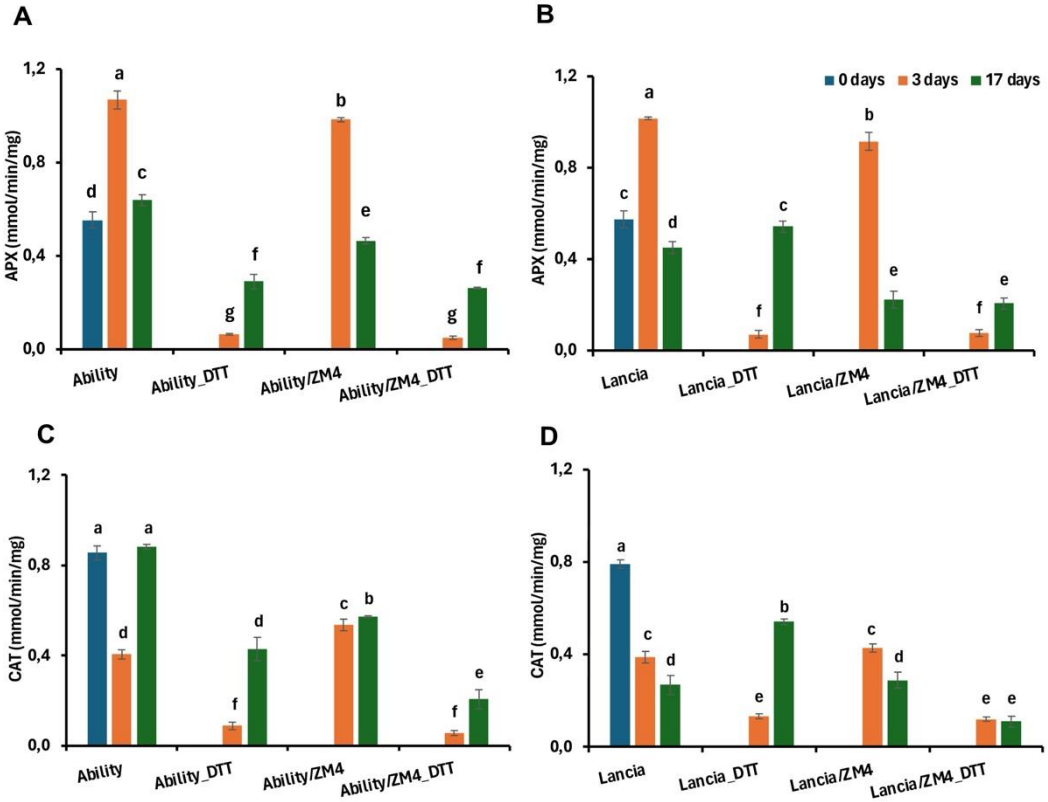

Figure 1 The impact of DTT and *Agrobacterium* infection on the enzyme activities of ascorbate peroxidase (APX) (**A, B**) and catalase (CAT) (**C, D**) in oilseed rape explants of cultivars Ability and Lancia, evaluated after 3 days and 17 days of cultivation in *in vitro* conditions. Data are mean ± SE. Different letters indicate statistically significant differences at $P \leq 0.05$.

APX and CAT are enzymatic antioxidants responsible for the decomposition of H_2O_2, albeit through distinct mechanisms (**Dumanović *et al.*, 2021; Li, 2023**). APX proves effective in handling lower levels of H_2O_2, and its activity may rise as the cellular antioxidant system begins adapting to stress. In contrast, CAT is efficient in rapidly eliminating elevated concentrations of H_2O_2 (**Hasanuzzaman *et al.*, 2020**). Correlation analysis revealed a positive relationship between the enzyme activities of APX and CAT in both cultivars (Table 1). The presence of DTT in the regeneration media on the third day led to a significant reduction (at least 7.4-fold) in APX activities in both *Agrobacterium*-infected (Ability/ZM4_DTT, Lancia/ZM4_DTT) and non-infected (Ability_DTT, Lancia_DTT) explants (Figure 1A, 1B). In contrast, regeneration without DTT led to a significant increase (at least 1.6-fold) in APX activities in both infected (Ability/ZM4, Lancia/ZM4) and non-infected (Ability, Lancia) explants. However, by the 17th day, APX activities exhibited a significant decrease (at least 1.7-fold) compared to the third day (Figure 1A). DTT-treated explants, in contrast, exhibited a significant increase in APX activity, at least 4.6-fold (Ability_DTT, Ability/ZM4_DTT) and at least 2.7-fold (Lancia_DTT, Lancia/ZM4_DTT). This increase might coincide with DTT's ability to scavenge hydrogen peroxide (**Chen and Asada, 1992**). DTT could potentially compete with ascorbate, which is a substrate of APX to reduce H_2O_2, resulting in a decline in APX activity. Regarding CAT activities, treatment with DTT led to a significant decrease of at least 5.9-fold in non-infected explants (Lancia_DTT and Ability_DTT) and at least 6.6-fold in *Agrobacterium*-infected explants (Lancia/ZM4_DTT and Ability/ZM4_DTT) (Figure 1C, 1D). On the third day, a reduction in CAT activity was also observed in DTT non-treated *Agrobacterium*-infected and non-infected explants, ranging from 1.6-fold (Ability/ZM4) to 2.1-fold (Ability, Lancia). On the 17th day of regeneration, CAT activities increased in DTT treated explants of the cultivars

Ability_DTT (4.9-fold), Lancia_DTT (4.1-fold), and Ability/ZM4_DTT (3.6-fold). CAT activities in DTT non-treated infected explants were unchanged (Ability/ZM4) or 1.5-fold diminished (Lancia/ZM4). In control explants, CAT activities either increased (2.2-fold, Ability) or decreased (1.5-fold, Lancia), possibly indicating a genotype-specific response. The variations in APX and CAT activity patterns between DTT-treated and DTT-non-treated explants could be ascribed to the direct interaction of DTT with H_2O_2, considering H_2O_2 as a substrate for both APX and CAT. DTT might function as a potential antioxidant in this context. Furthermore, the specific roles of CAT and APX in the cellular antioxidant system need to be considered as well. Several authors indicate that DTT, when used during *Agrobacterium* infection, can reduce ROS accumulation, thereby potentially increasing transformation efficiency (**Li *et al.*, 2017; Zhao *et al.*, 2020**).

Proline is recognized as a multifunctional amino acid with the ability to serve as a metal chelator, antioxidant, osmoprotectant, or stress signaling molecule in plant growth and development (**Szabados and Savoure, 2010; Ghosh *et al.*, 2021**). It plays a crucial role in cell division and elongation, which are essential for the successful regeneration of plant tissues in culture (**Kishor *et al.*, 2015**). The proline contents in explants significantly decreased by at least 2.8-fold on the third day in regeneration media for Ability, Ability_DTT, and Ability/ZM4_DTT, while remaining unchanged for Ability/ZM4, Lancia, Lancia/ZM4, Lancia_DTT, and Lancia/ZM4_DTT (Figure 2A, 2B). In contrast, on the 17th day, proline contents significantly increased in both DTT-treated and non-treated explants of both cultivars. Specifically, in DTT-treated explants of the cultivar Ability, the proline content increased by at least 20.4-fold (Ability_DTT and Ability/ZM4_DTT), while in DTT non-treated explants, it was at least 2.5-fold (Ability and Ability/ZM4). For the cultivar Lancia, in DTT-treated explants, the proline content increased by 10.9-fold (Lancia_DTT) and 7.4-fold (Lancia/ ZM4_DTT), while in DTT-non-treated explants, it increased by 14.6-fold (Lancia) and 5.4-fold (Lancia/ZM4). It seems that proline might play an important role in the *in vitro* culture of oilseed rape, and its accumulation appears to be linked to its multifaceted role in plant tissue culture. Several authors suggested that the addition of exogenous proline to regeneration media can enhance the *in vitro* regeneration process (**Pawar *et al.*, 2015**; **Pazuki *et al.*, 2018**). On the other hand, plant cells can accumulate proline not only in response to abiotic stress conditions (**Meena *et al.*, 2019**) but also during pathogen infection, including *A. tumefaciens*. **Haudecoeur *et al.* (2009)** pointed out the antagonist role of proline in the synthesis of gamma-aminobutyric acid (GABA) which is a part of plant defense against *Agrobacterium.* To some extent, the elevated level of proline might potentially counterbalance the inhibitory effect of DTT on the functionality of antioxidant enzymes. Correlation analysis showed a negative trend between proline content and APX/CAT activities (Table 1).

Table 1 Correlation analysis between APX, CAT, proline, and carbohydrates in explants of the cultivars Ability and Lancia

	Ability				Lancia			
	APX	CAT	PROL	TSS	APX	CAT	PROL	TSS
APX	-				-			
CAT	0,510*	-			0,536*	-		
PROL	-0,262	-0,125	-		-0,123	-0,179	-	
TSS	-0,103	-0,650*	0,131	-	-0,031	-0,694*	0,421	-

*Significant at p<0.05; APX – ascorbate peroxidase, CAT – catalase, PROL – proline, TSS– total soluble sugars

Soluble sugars function as multifunctional osmoprotectants, participating in osmotic adjustment, water retention, stabilization of cellular structures, antioxidant functions, regulation of gene expression during stress, and providing energy for crucial metabolic processes (**Rosa *et al.*, 2009**; **Ghosh *et al.*, 2021**). The total soluble content increased by at least 2.4-fold in both DTT-treated and non-treated, as well as *Agrobacterium*-infected explants of both cultivars, after three days of regeneration (Figure 2C, 2D). On the 17th day of regeneration, the total soluble content significantly increased by at least 1.3-fold (Ability/ZM4, Ability/ZM4_DTT, and Lancia/ZM4_DTT), remained unchanged (Ability, Lancia_DTT, and Lancia/ZM4), or decreased by at least 1.4-fold (Lancia, Ability_DTT), compared to the third day. The observed changes in the total soluble sugar content might be considered one of the strategies that plants employ, depending on the genotype, to cope with osmotic imbalances resulting from *in vitro* culture conditions. Furthermore, the pathogenicity of *Agrobacterium* is primarily associated with its ability to respond to chemical signals originating from damaged plant cells, including released sugars (**Subramoni *et al.*, 2014**). The negative correlation between CAT activities and soluble sugar contents (Table 1) indicates the interplay among metabolic regulation, and antioxidant mechanisms aimed at preserving cellular homeostasis.

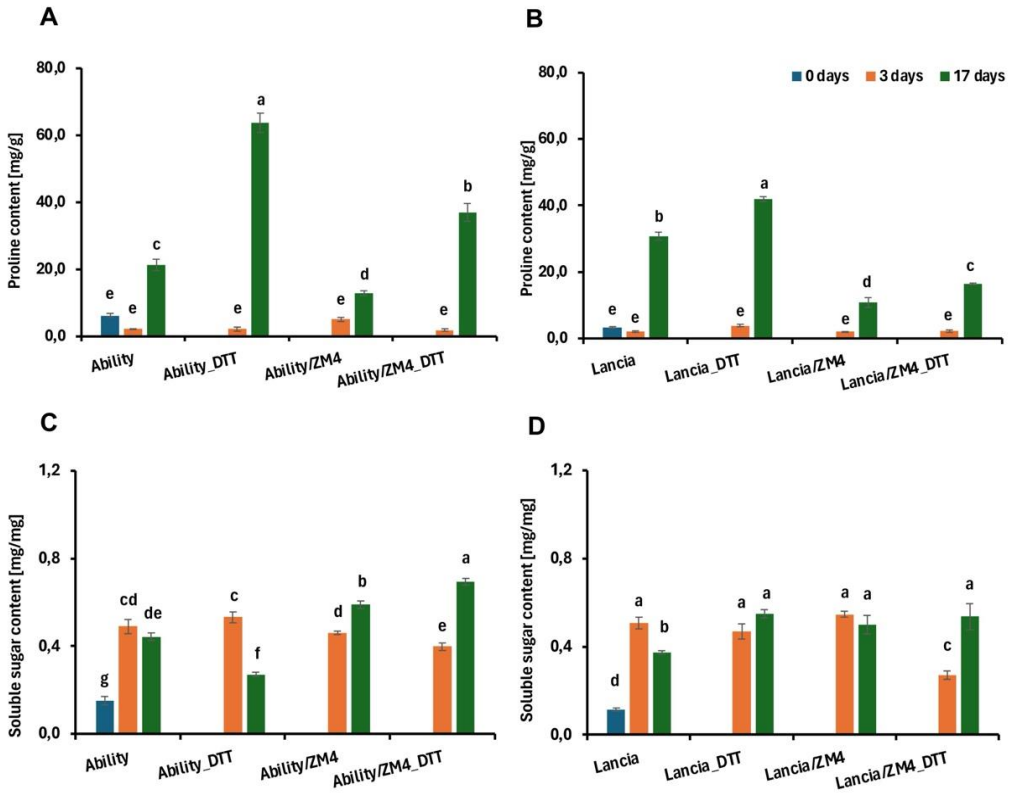

Figure 2 The impact of DTT and *Agrobacterium* infection on the proline content (**A, B**) and total soluble sugar content (**C, D**) in oilseed rape explants of the cultivars Ability and Lancia, evaluated after 3 days and 17 days of cultivation in *in vitro* conditions. Data are mean ± SE. Different letters indicate statistically significant differences at $P \leq 0.05$.

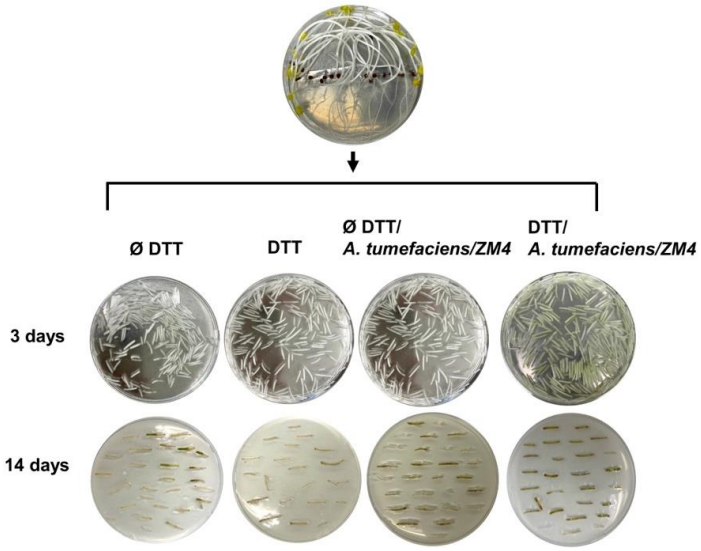

Figure 3 An example of *in vitro* regeneration of *Agrobacterium* infected/non-infected explants of the cultivar Ability cultured for 3 days in the liquid regeneration medium and then 14 days on solid regeneration in the presence and absence (Ø) of DTT.

CONCLUSION

Our results revealed that the application of DTT at a concentration of 1 mg/L in the culture media significantly influenced the activities of enzymatic antioxidants, including APX and CAT, as well as the content of the nonenzymatic antioxidant and osmoprotectant proline in both *Agrobacterium*-infected and non-infected hypocotyl segments from both cultivars (Ability and Lancia). After 3 days of *in vitro* culture, *Agrobacterium*-infected and non-infected explants responded to the presence of DTT with a significant decrease in the activities of both enzymes, APX and CAT. In contrast, explants regenerated without DTT exhibited a significant increase in APX activity and a decrease in CAT activity; however, the decrease in CAT activity was not as pronounced as observed in DTT-treated non-infected as well as *Agrobacterium*-infected explants. On the 17th day of *in vitro* culture, we observed an opposite APX activity pattern. APX activity significantly increased in both DTT and DTT/*Agrobacterium* treated explants, while in non-infected and *Agrobacterium*-infected explants regenerated without DTT, the activity of APX significantly decreased. It indicates that the components of the antioxidant system might engage in defending against ROS at different times and levels, seemingly adhering to the principle of mutual compensation. The proline content increased significantly in all analysed explants, but the most in explants regenerated in the presence of DTT. This suggests that DTT might indirectly contribute to the reduction of ROS generation, thereby improving the infectivity of *Agrobacterium* and, consequently, increasing the transformation efficiency.

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