JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal
Published by Faculty of Biotechnology and Food Sciences

EFFECT OF SILVER NITRATE ON *IN VITRO* REGENERATION AND ANTIOXIDANT RESPONSES OF OILSEED RAPE CULTIVARS (*BRASSICA NAPUS* L.)

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https://doi.org/10.15414/jmbfs.4494

ARTICLE INFO

Received 11. 3. 2021
Revised 19. 5. 2021
Accepted 19. 5. 2021
Published 1. 6. 2021

Regular article

OPEN ACCESS

ABSTRACT

We studied a stimulatory effect of silver nitrate on callus formation and shoot regeneration of five oilseed rape commercial varieties Ability, Lagonda, Lancia, Menthal and Mirakel (*Brassica napus* L.). Hypocotyls were induced to organogenesis on the media supplemented with silver nitrate at concentration 5 mg.L^{1} and without silver nitrate as a control. After 2 weeks of cultivation, calli on the media with silver nitrate became deep green in colour. First shots appeared after 4 weeks of cultivation on the media with silver nitrate. Calli formed on the control media were pale green and did not form any shoots or at very low efficiency. Silver nitrate improved regeneration efficiency from 0- 0.8% to 8.3-10% in varieties Albina, Lancia and Menthal. Explants of Lagonda and Mirakel did not form any calli and these varieties appeared to be recalcitrant. Analyses of six weeks-old calli showed that silver nitrate increased concentration of photosynthetic pigments, which is a good prerequisite for cell regeneration. Silver nitrate increased malondialdehyde level and induced changes in enzyme activities of antioxidants ascorbate peroxidase and catalase; and in the accumulation of non-enzymatic antioxidant proline; in the genotype dependant manner. The results suggest that an activation of stress response was necessary to obtain shoots with higher frequency in the varieties Albina, Lancia and Menthal.

Keywords: ascorbate peroxidase, *Brassica napus L.*, catalase, *in vitro*, MDA, photosynthetic pigments, proline, silver nitrate

INTRODUCTION

Oilseed rape (*Brassica napus* L.) as one of the most important sources of vegetable oil has been of interest to breeders for many years. Conventional breeding programmes have been mainly attempted to produce oilseed rape varieties with modified content of fatty acids. The main obstacles to the application of modern methods such as gene transfer are a highly variable *in vitro* regeneration efficiency and genotype specificity of oilseed rape (**Park *et al.*, 2012**). Until now, only the genetically modified Argentine Canola has been authorized (**https://www.isaaa.org, 2021**).
Despite advances in tissue cultures, genotype specificity is a major limiting factor (**Bhowmik *et al.*, 2011; Farooq *et al.*, 2019**). Some oilseed rape cultivars remain recalcitrant to *in vitro*. Ethylene accumulation, high humidity in culture vessels, nutrient-rich media or high doses of cytokinins and auxins appear to be a serious problem for regeneration of oilseed rape (**Park *et al.*, 2012; Paladi *et al.*, 2017; Farooq *et al.*, 2019**).
Ethylene is a plant hormone that regulates many developmental processes and modulate stress responses (**Polko and Kieber, 2019**). An accumulation of ethylene in tissue cultures is often accompanied by reduced growth and morphological changes (**Roshanfekrrad *et al.*, 2017; Tahoori *et al.*, 2018**). Moreover, ethylene is associated with formation of reactive oxygen species (ROS) (**Zhang *et al.*, 2016**). An overproduction of ROS leads to oxidative stress and programmed cell death (**Dan *et al.*, 2008; Baťková *et al.*, 2008; Bidabadi and Jain, 2020**). ROS are regulated by cellular defence system consisting of enzymatic (superoxide dismutase, ascorbate peroxidase, catalase, glutathione reductase, etc.) and non-enzymatic (ascorbic acid, proline, carotenoids, etc.) antioxidants. Maintaining of ROS at optimum level enables regulate numerous cell processes during plant growth and development. Overproduction of ROS causes damage of DNA, proteins or lipids (**Gill and Tuteja, 2010; Soares *et al.*, 2019; Hasanuzzaman *et al.*, 2020**).

$AgNO_3$ plays dual role in tissue cultures. At low concentration, $AgNO_3$ stimulates plant regeneration while at higher concentration promotes oxidative stress (**Qin and Zhang, 2005; Baťková *et al.*, 2008; Barbasz *et al.*, 2016**). $AgNO_3$ is considered an ethylene inhibitor in *in vitro* culture of many plant tissues (**Ghobeishavi *et al.*, 2015; Sarropoulou *et al.*, 2016; Jaberi *et al.*, 2018; Tahoori *et al.*, 2018**). Its inhibitory effect consists in preventing of ethylene-induced plant responses by substituting Cu ions in ethylene receptors (**Zhao *et al.* 2002; Kumar *et al.*, 2009; McDaniel and Binder, 2012**). Moreover, $AgNO_3$ can reduce production of 1-aminocyclopropane-1-carboxylic acid, a direct precursor of plant ethylene (**Prem Kumar *et al.*, 2016**). As ethylene and polyamines share a common precursor S-adenosylmethionine synthetase, reduction in ethylene production leads to increased polyamines formation (**Prem Kumar *et al.*, 2016; Asgher *et al.*, 2018**). Polyamines stimulate cell division and plant growth (**Park *et al.*, 2012; Prem Kumar *et al.*, 2016**).
We aimed to investigate a stimulatory effect of $AgNO_3$ at concentration of 5 mg.L^{-1} on shoot organogenesis of five commercial oilseed rape cultivars Ability, Lagonda, Lancia, Menthal and Mirakel. At the same time, we studied biochemical effects of $AgNO_3$ on callus formation.

MATERIAL AND METHODS

Plant material and explant preparation

Mature seeds of spring oilseed rape (*Brassica napus* L.) cultivars Ability, Lagonda, Lancia, Menthal, and Mirakel were obtained from Norddeutsche Pflanzenzucht, Hohenlieth-Hof, Germany. Surface-sterilized seeds (**Boszorádová *et al.*, 2011**) were germinated on MS medium (**Murashige and Skoog, 1962**) with 1% (w/v) sucrose, 0.7% (w/v) agar, pH 5.8 at 23°C in darkness for 6 days.

Plant regeneration

Regeneration of hypocotyl explants was performed according to **Boszorádová *et al.* (2011)** with modification. Six days old hypocotyls were cut into 5-10 mm long segments and pre-incubated in liquid callus inducing (CIM) medium [Gamborg B5 medium, 2% (w/v) sucrose, 250 mg.L^{-1} NH_4NO_3, 750 mg.L^{-1} $CaCl_2 \cdot 2\ H_2O$, 250 mg.L^{-1} xylose, pH 5.8] for 1 hour. Then, segments were transferred to solid CIM medium supplemented with 1 mg.L^{-1} 2.4 D and 0.1 mg.L^{-1} IAA, 0.6% (w/v) agar; and with or without $AgNO_3$ (5 mg.L^{-1}). Following 2 weeks, segments were transferred to shoot-inducing (SIM) medium [Gamborg B5 medium, 2% (w/v) sucrose, 250 mg.L^{-1} NH_4NO_3, 750 mg.L^{-1} $CaCl_2 \cdot 2\ H_2O$, 250 mg.L^{-1} xylose, 2 mg.L^{-1} BAP, 1 mg.L^{-1} zeatin, 0.6% (w/v) agar, pH 5.8] with or without $AgNO_3$ (5 mg.L^{-1}). Developed shoots were excised and transferred to shoot-elongation (SEM) medium [½ strength Gamborg B5 medium, 500 mg.L^{-1}, 2-(*N*-morpholino)ethanesulfonic acid, 1% (w/v) sucrose, pH 5.8, 0.6% (w/v) agar]. The experiments were performed in 3 biological replicates (45 explants/replicate). Explants (15 explants/Petri dish/cultivar) were incubated at 23°C and 16 h/8 h light/dark photoperiod under 50 µE m^{-2}.s^{-1} light intensity.

Callus producing efficiency (C_{PE}) was evaluated in % as the number of explants forming calli to the total number of explants. Shoot producing efficiency (S_{PE}) was evaluated in % as the number of developed shoots to the total number of explants.

Biochemical effects of $AgNO_3$ on callus formation

Analyses were performed on callus tissues collected after six weeks of cultivation on regeneration media supplemented with $AgNO_3$ or without $AgNO_3$. Callus tissues were pooled and sampled (3 samples per treatment per cultivar per assay). Samples were frozen in liquid N_2 and stored at -80ºC until analyses.

The content of chorophyll *a*, chlorophyll *b* and carotenoids was measured by the method **Lichtenthaller and Wellburn (1983).** The content of malondialdehyde (MDA) was determined according to **Karabal *et al.* (2003)** and evaluated in nmol.g^{-1} of fresh weight (FW). Activities of antioxidant enzymes ascorbate peroxidase (APX) and catalase (CAT) were measured by the method **Kováčik *et al.* (2009).** Protein content was assayed according to **Bradford (1976).** Enzyme activities of APX and CAT were expressed in mmol.min^{-1}.mg^{-1} of soluble proteins and µmol.min^{-1}.mg^{-1} of soluble proteins, respectively. Concentration of non-enzymatic antioxidant proline was measured via reaction with ninhydrin (**Paquin and Lechasseur, 1979**) and expressed in mg.g^{-1} of FW.

Statistical analyses

Data were evaluated using statistical functions in Microsoft Excel 2013. Analysis of variance together with a post hoc test (Tukey's test) were performed using SPSS Statistics 22 software (IBM).

RESULTS AND DISCUSSION

We studied a stimulatory effect of $AgNO_3$ on callus formation and shoot organogenesis of five oilseed rape varieties Ability, Lagonda, Lancia, Menthal and Mirakel. Hypocotyls from 5-days old seedlings were used as a source of explants (Figure 1A). The explants were regenerated on the media supplemented with $AgNO_3$ (Ag+) and without $AgNO_3$ (Ag-). The concentration 5 mg.L^{-1} of $AgNO_3$ was chosen based on our previous experiences (**Boszorádová *et al.*, 2011**) and on the literature data (**Schröder *et al.*, 1994; Uliaie *et al.*, 2008; Farooq *et al.*, 2019; Jiang *et al.*, 2020**). $AgNO_3$ (5 mg.L^{-1}) is standardly used in *in vitro* regeneration of oilseed rape. The number of calli and shoots were evaluated as a percentage at two-week intervals for a period of 6 weeks. Data are summarized in Figure 2.

The hypocotyls of two out of five studied varieties Lagonda and Mirakel did not respond to regeneration conditions. The explants turned brown approximately after one week of cultivation on both (Ag+) or (Ag-) media and did not produce any calli (Figure 1B). Tissue browning is usually attributed to the oxidation of phenolic compounds to quinones (**Huang *et al.*, 2002; Jones and Saxena, 2013; Wang *et al.*, 2016**). The oxidation reaction might be induced by stress caused by e.g. cultivation conditions (**Wang *et al.*, 2016**).

Unlike Lagonda and Mirakel, given cultivation conditions allowed to regenerate hypocotyl-derived calli of varieties Ability, Lancia and Menthal. First calli were yellow pale in colour and appeared after one week of cultivation regardless of $AgNO_3$. Then, calli became gradually greenish (Figure 1B). After 2 weeks, explants formed green calli with the efficiency from 15.8% to 39.2% (Ag+) and from 7.5% to 40.8% (Ag-) (Figure 2). The positive effect of $AgNO_3$ on the callus induction was observed in the variety Menthal. After 4 weeks of cultivation on (Ag+) media, the number of green calli (Menthal) was at least 1.6 fold higher than on the (Ag-) media.

Calli cultivated on the (Ag+) media were deep green in colour (Figure 1E) while calli on the (Ag-) media were pale green (Figure 1D). The first shoots appeared after 4 weeks on the (Ag+) media (Figure 1E). Developed shoots were transferred to the elongation medium (Figure 1F). An efficiency in shoot production ranged from 8.3% (Ability, Menthal) to 10% (Lancia) (Figure 2). Pale green calli did not form any shoots (Ability, Menthal) or at very low efficiency (0.8%, Lancia). Thus, an application of $AgNO_3$ at concentration of 5 mg.L^{-1} showed higher ability of calli to produce shoots.

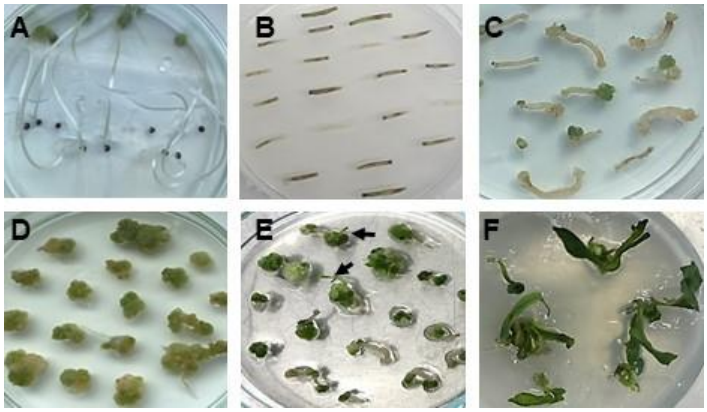

Figure 1 An example of *in vitro* regeneration of oilseed rape (*Brassica napus* L.). **A** Five days-old seedling used as an explant source. **B** Hypocotyl segments of the variety Lagonda after 2 weeks on the regeneration medium. **C** Callus formation after 2 weeks on the regeneration medium supplemented with $AgNO_3$. **D** Callus formation after 4 weeks on the regeneration medium without $AgNO_3$. **E** Callus formation after 4 weeks on the regeneration medium supplemented with $AgNO_3$. Arrows indicate formation of the first shoots. **F** Shoots on the elongation medium.

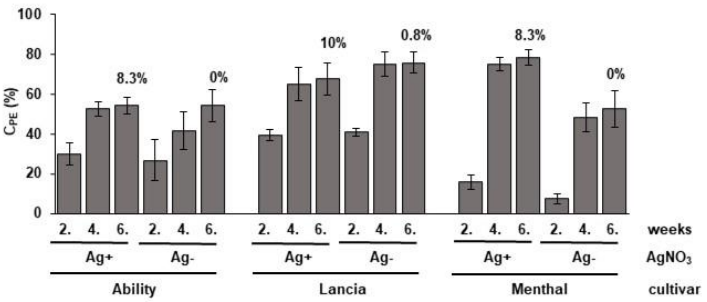

Figure 2 Effect of $AgNO_3$ on callus (C_{PE}) and shoot (S_{HE}) producing efficiencies in the varieties Ability, Lancia and Menthal.

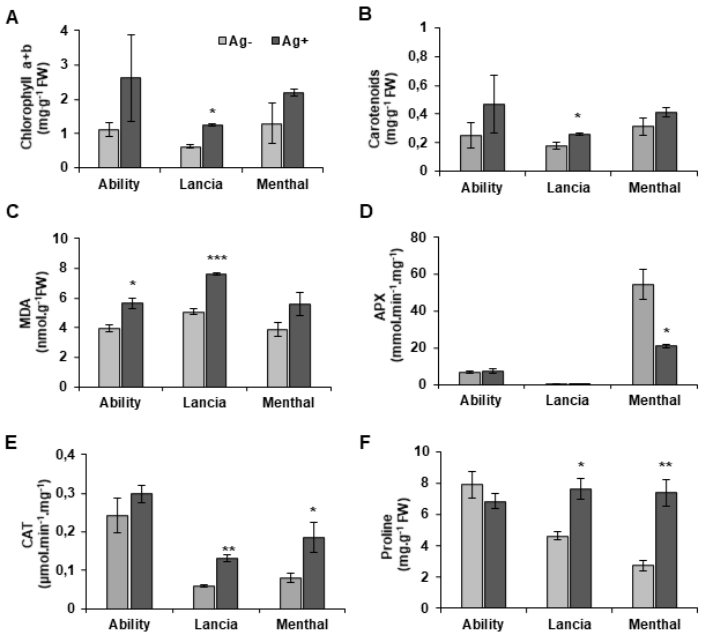

Figure 3 Effects of $AgNO_3$ on the concentration of total chlorophyll (**A**), carotenoids (**B**), malondialdehyde level (MDA) (**C**), enzyme activities of ascorbate peroxidase (APX) (**D**) and catalase (CAT) (**E**); and accumulation of proline (**F**) in 6 weeks-old calli of the varieties Ability, Lancia and Menthal; * statistically

significant at p<0.05, ** statistically significant at p<0.01, *** statistically significant at p<0.001. Data are mean ± SEM.

Developed calli were deep and pale green in colour [(Ag+) and (Ag-) media, respectively] (Figure 1). Analyses of calli after 6 weeks of cultivation on the $AgNO_3$ containing media revealed that the concentration of total chlorophyll (chl*a*+chl*b*) (Figure 3A) was at least 1.3 fold higher compared to the control (Ag-) calli. Similarly, the concentration of carotenoids (Figure 3B) was at least 1.7 fold higher compared to the control (Ag-) calli. In addition to their function in photosynthesis, carotenoids also act as antioxidants (**Gill and Tuteja, 2010**). Although, $AgNO_3$ had a positive effect on the content of photosynthetic pigments in calli of all varieties, this effect was statistically significant (at p<0.05) only for the variety Lancia. It may be related to the fact that we analyzed calli, a mass of cells with different level of differentiation. The genotype did not significantly affect (at p<0.05) the concentration of photosynthetic pigments (Table 1).

Tissue cultures are accompanied by a higher production of ROS **(Gupta, 2010).** Malondialdehyde (MDA) is one of the products of peroxidation of polyunsaturated fatty acids of membranes and its increased concentration is considered a common manifestation of oxidative stress in plants (**Sharma *et al.*, 2012**). In our study, the treatment with $AgNO_3$ caused a significant increase (at least 1.4 fold) in MDA accumulation in calli of all varieties (Figure 3C). The MDA level in (Ag-) calli was not statistically (at p<0.05) different between varieties (Table 1).

The plant cells respond to increased levels of ROS through an antioxidant mechanism that involves the production of enzymatic and non-enzymatic antioxidants (**Gill and Tuteja, 2010; Soares *et al*., 2019; Hasanuzzaman *et al*., 2020**). We measured the activities of enzymes ascorbate peroxidase (APX), catalase (CAT) (Figure 3D, 3E) and the content of proline (Figure 3F). APX and CAT are enzymatic antioxidants that break down H_2O_2, but in a different way **(Soares *et al*., 2019; Hasanuzzaman *et al*., 2020**). H_2O_2 plays dual role, at low concentrations acts as a key regulator of many physiological processes and at high concentrations leads to programmed cell death (PCD) (**Gill and Tuteja, 2010**). We did not observe changes in APX activity upon exposure of $AgNO_3$ in calli of the varieties Ability and Menthal (Figure 3D). The activity of APX in the (Ag+) calli of the variety Menthal has been significantly (at p <0.05) reduced, which may be related to the genotype (Figure 3D, Table 1).

$AgNO_3$ induced CAT activity in the varieties Lancia and Menthal (Figure 3E). CAT activity was significantly (at p <0.05) higher at least 2.2 fold compared to their counterparts. We did not detect significant differences in CAT activity between (Ag+) and (Ag-) calli of the variety Ability. However, the activity of CAT in (Ag-) calli was significantly higher (at p <0.05) compared to the (Ag-) calli of the varieties Lancia and Menthal (Figure 3E, Table 1).

We found that $AgNO_3$ significantly (at least 1.6 fold, at p <0.05) increased the level of proline in calli of the varieties Lancia and Menthal but no in the variety Ability (Figure 3F). However, the accumulation of proline in the (Ag-) calli of the variety Ability has been significantly higher (at p <0.05) compared to other genotypes (Table 1). Proline acts as an osmoprotectant, ROS (·OH) scavenger and potential inhibitor of PCD (**Liang *et al*., 2013; Soares *et al*., 2019**). Moreover, accumulation of proline in green tissues might avoid photo-inhibition **(Signorelli, 2016**).

Table 1 Total chlorophyll, carotenoids concentrations; malondialdehyde (MDA) content; ascorbate peroxidase (APX) and catalase (CAT) enzyme activities and proline accumulation as affected by genotype.

	Total chlorophyll [mg.g^{-1} FW]	Carotenoids [mg.g^{-1} FW]	MDA [nmol.g^{-1} FW]	APX [mmol.min^{-1}.mg^{-1}]	CAT [µmol.min^{-1}. mg^{-1}]	Proline [mg.g^{-1} FW]
Ability	1.095±0.201 *a*	0.249±0.087 *a*	3.978±0.228 *a*	6.765±0.844 *a*	0.243±0.045 *b*	7.888±0.877 *b*
Lancia	0,621±0.046 *a*	0.179±0.022 *a*	5.054±0.197 *a*	0.642±0.045 *a*	0.059±0.004 *a*	4.627±0.249 *a*
Menthal	1,291±0.059 *a*	0.311±0.060 *a*	3.871±0.773 *a*	54.231±8.199 *b*	0.080±0.011 *a*	2.736±0.311 *a*

Analyses were performed using the single factor ANOVA with Tukey's post hoc test. Different letters of alphabet mean statistical significance between genotypes at p<0.05. Data are mean ± SEM.

CONCLUSION

Our results showed that an application of $AgNO_3$ at concentration 5 mg.L^{-1} in regeneration media increased shoot regeneration efficiency from 0-0.8% to 8.3-10% in varieties Albina, Lancia and Menthal. Varieties Lagonda and Miracel appeared to be recalcitrant. Analysis of six weeks old callus tissues showed that $AgNO_3$ had a stimulatory effect on the concentration of photosynthetic pigments. $AgNO_3$ activated ROS and induced MDA level in all varieties. We observed genotype-dependent differences in enzyme activities of APX, CAT and accumulation of proline. $AgNO_3$ increased activity of CAT and proline content in the varieties Lancia and Menthal. The effect of $AgNO_3$ on enzyme activities APX, CAT and proline in the variety Ability was not statistically significant. Our results suggest that a stress response triggered by $AgNO_3$ might contribute to higher frequency of shoot regeneration in the varieties Albina, Lancia and Menthal.

Acknowledgments: This work was supported by findings from the grant agencies APVV-16-0051 and VEGA 1/0525/20; and FPPV-33-2021.

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