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Cold-induced Changes of Antioxidant Enzymes Activity and Lipid Peroxidation in Two Canola (*Brassica napus* L.) Cultivars

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Abstract
This study was conducted on two canola (*Brassica napus*) cultivars, Okapi a winter type and cold tolerant and Rgs003 a spring type and cold sensitive. Seedlings were grown in an environmentally controlled growth room with 16 h d^{-1} photoperiod at 22/16 °C (day/night, control). At the 4-leaf stage, half of pots were transferred to a cold growth room for 7 d at 10/3°C (day/night, cold treatment) and their leaves were harvested as required on 0, 2, 4 and 7 d as four sampling times. Superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX) activity and the amount of malondialdehyde (MDA) in samples were assessed. Analysis of variance showed that winter canola had more SOD, CAT and APX activity, lower amount of either MDA or POD activities compared with the spring canola. This study indicated that antioxidant mechanisms caused more cold tolerance in the winter cultivar of canola compared with the spring cultivar.

Keywords: Antioxidant enzymes (APX, CAT, POD, SOD); Canola; Cold stress; Lipid peroxidation

Abbreviations: APX - ascorbate peroxidase; CAT - catalase; H_2O_2 - hydrogen peroxide; LT – low temperature; MDA - malondialdehyde; POD - peroxidase; ROS - reactive oxygen species; SC - spring canola; SOD - superoxide dismutase; WC - winter canola

Introduction

Oilseeds are the second world food stores after cereals. Among which, canola is a common annual oil crop in cold and mild cold regions and after soybean and cotton is the third world oil crop (FAO 2011). In Iran, canola is edible oil crop that has recently been exploited to boost its production. On the other hand, low temperature is one of the most important abiotic factors limiting growth, productivity and distribution of plants (Nordin *et al*. 2003). Low temperature (LT) decreases the biosynthetic activity of plants and inhibits the normal function of their physiological processes and may cause permanent injuries, finally leading to death (Zhu *et al*. 2007). Most crops are exposed to fluctuant and seasonal low temperature that limits their photosynthesis, respiration and growth even in their natural growth habitats (Boyer 1982). The effect of cold stress on plants life has been studied for several times and many attempts have been made to improve plant cold tolerance. Various mechanisms have been suggested to estimate cold tolerance in plants, e.g. cellular accumulation of ROS which can damage proteins, membrane lipids and nucleic acids. ROS, such as superoxide radicals (O_2^-), H_2O_2, hydroxyl radicals (OH^-), and singlet oxygen ($^-O_2$), are elevated under stress

conditions (Mittler 2002; Guo *et al*. 2003). Plants have developed the ROS scavenging mechanism to protect cellular membranes and organelles from damaging effects of ROS (Bian and Jiang 2009). Plant's major ROS-scavenging enzymes include SOD, APX, CAT and POD. The balance between SOD and APX or CAT activities in cells is a crucial point to maintain the steady-state level of ROS (Kwon *et al*. 2001). Generally, as the main cell antioxidant mechanism, it is proven that in the first step SOD catalyzes the dismutation of O_2^- to H_2O_2 and O_2 molecules. Then, H_2O_2 is detoxified by APX, POD and CAT in different organells and antioxidant cycles (Mittler 2002). Several assays show that defensibility against oxidative damages is inhibited by the reduction of antioxidants expression such as SOD, CAT, POD and APX. Cold tolerance is improved when plant's POD, CAT and SOD levels enhance. Studies determined that the activity of antioxidant enzymes is correlated with plant tolerance to cold stresses, such as responses to cold stress in wheat (Javadian *et al.* 2010), strawberry (Luo *et al*. 2011) and barley (Radyuk *et al*. 2012).

In addition, the degree of damage by ROS depends on the balance between the product of ROS and its removal by this antioxidant scavenging mechanism (Azooz *et al*. 2009). Damage to fatty acids of membrane by an uncontrolled enhancement of free radical could then produce small hydrocarbon fragments including MDA (Moussa and Aziz 2008). MDA is the final product of plant cell membrane lipid peroxidation and is one important sign of membrane system injury (Cunhua *et al*. 2010). It is important to calculate antioxidant enzymes activity during cold treatment to determine their rolls in making cold tolerance (Xu *et al*. 2008). Thus, this study was aimed to examine the effect of short-term cold stress on antioxidant enzymes (APX, CAT, POD, SOD) activities and the amount of lipid peroxidation (by measuring the amount of MDA) in seedlings of two (cold-tolerant and cold-sensitive) canola cultivars.

Materials and Methods

Plant material and cold stress induction

Seeds of two canola (*Brassica napus* L.) cultivars, Okapi as a winter type and cold tolerant and Rgs003 as a spring type and cold sensitive were sown in ready pots, containing a soil mixture of sand: peat: vermiculate (1:1:1) in an environmentally controlled growth room programmed for a photoperiod of 16 h d^{-1} at a light intensity of 300 μmol m^{-2} s^{-1} at soil level at 22/16 ºC (day/night, controls). For cold exposure, half of the pots containing young plants at four-leaf stage were transferred to a cold growth room set at the same light intensity and photoperiod for 7 d at 10/3 ºC (day/night, cold treatment). This time of transferring to cold treatment was considered as experimental day 0. Therefore, fresh leaf samples of control and cold-treated seedlings were harvested and frozen in liquid nitrogen on experimental days (0, 2, 4 and 7) of exposure to cold treatment as four sampling times to determine antioxidant enzymes activities.

Estimation of antioxidant enzymes

The amount of 0.2 g frozen samples were homogenized using ice cold mortar and pestle in 3 ml of HEPES-KOH (pH 7.8) buffer with 0.1 mM EDTA to determine SOD activity. The homogenate was centrifuged at 15000 rpm for 15 min at 4 ºC. The supernatant was used as a source of SOD enzyme. SOD activity was measured by a photochemical method (Gianaopolitis and Reis 1977). The reaction mixture (3 ml) contained 0.1 mM EDTA, 50 mM HEPES-KOH buffer (pH 7.8), 50 mM Na_2CO_3 (pH 10.2), 12 mM L-methionine, 75 NBT, 300 μl enzyme extract and 1 μM riboflavin. The absorbance was read at 560 nm and one unit activity of SOD was defined as the rate of enzyme required to result in a 50% inhibition of rate of NBT reduction. CAT activity was measured by the method of Cakmak and Horsrt (1991). The reaction mixture consisted of 2.6 ml of 25 mM Na-phosphate buffer (pH 6.8), 400 μl of 10 mM H_2O_2 and 40 μl of enzyme. The decomposition of H_2O_2 was followed by the decline of absorbance at 240 nm. APX activity was determined by a method of Nakano and Asada (1981). The 0.2-gram samples were homogenized in 1 ml of 50 mM Na-phosphate buffer (pH 7.8) containing 0.1 mM EDTA, 5 mM ascorbate, 5 mM DTT, 100 mM NaCl and 2% (w/v) PVP. The homogenate was centrifuged at 15000 rpm for 15 min at 4 ºC. The supernatant was used as a source of enzyme. The reaction was initiated by adding H_2O_2 to a solution with final concentration of 44 μM. The decrease in absorbance was monitored at 290 nm. The rate of APX was calculated using the extinction coefficient of 2.8 $mM^{-1}cm^{-1}$ and correction was done for the non-enzymatic oxidation of ascorbic acid that obtained prior to addition H_2O_2. The activity of POD was determined in a reaction mixture which consisted of suitable amount of 28 mM guaiacol, 5 mM H_2O_2, 25 mM Na-phosphate buffer (pH 6.8) and enzyme (Ghanati *et al*. 2002). Soluble protein content was estimated by the method of Bradford (1976), and BSA used as a standard.

Estimation of lipid peroxidation

Lipid peroxidation was determined by measuring MDA content. The 0.2-gram frozen samples were homogenized in 3 ml of 10% (v/v) trichloroacetic acid. The homogenate was passed through filter paper and added 1 ml of 0.5% (v/v) thiobarbituric acid (TBA) solution to each extraction. The final extraction was heated in a boiling water bath at 100ºC for 30 min and allowed to cool in ice bath. The absorbance of solution was recorded at 532 nm followed by correlation for the nonspecific absorbance at 600 nm. The amount of MDA was determined according to extinction coefficient of 155 mM^{-1} cm^{-1} as described by De Vos *et al*. (1991).

Statistical analysis

The data were first tested for normality and then analyzed as balanced 3-factor analysis of variance (ANOVA) based on completely randomized design (CRD) with three replications, using Minitab 16 statistical software (Fry 1993; Ryan and Joiner 2001). Cultivars, temperature treatments and sampling times were considered as

three factors with 2, 2 and 4 levels, respectively. Means and standard errors (SE) were used to compare temperature treatments within each cultivar at each sampling time, using LSD.

Result and Discussion

Effect of cold on antioxidant enzymes

The result of ANOVA is shown in Table 1. The treatment and treatment × sampling time interaction for the three antioxidant enzymes activities (SOD, POD, CAT) were significant (P<0.001), which indicated noticeable differences between two canola cultivars in various sampling times. For APX and MDA, the same was true for between-treatment differences but not for the interaction. SOD is a crucial enzyme in oxidative stresses such as cold stress and it is the only antioxidant enzyme that inactivates superoxide radical (Kwon *et al*. 2001). Interestingly, cold treatment caused enhanced SOD activity in cold-induced leaves of both canola cultivars, compared with the control (Figure 1, WC). Okapi as a cold tolerant cultivar demonstrated higher activity than Rgs003 as a spring cold sensitive cultivar (Figure 1, SC). In other words, on day 7 of cold exposure, the activity of SOD enhanced by 2.5-fold (P<0.001) in WC cold-treated leaves compared with those of the controls: to a lesser extent, it was true for SC cultivar at this sampling time (1.7-fold increase, P<0.001). This is coincident with other reports emphasizing the crucial role of SOD under oxidative stress (McCord 1988; Erdal *et al.* 2012, Rajabi *et al.* 2012). In addition, Wang-Hao *et al.* (2007) proved that canola SOD isoforms were enhanced under cold treatment. A similar cold response was detectable for CAT activity

Table 1. Mean squares of the 3-factor ANOVA on the basis of completely randomized design for five physiological characteristics in two canola cultivars

SOV	df	MS				
		SOD	POD	CAT	APX	MDA
Cultivar (cv.)	1	0.494^{ns}	1.333^{**}	0.145^{ns}	29.435^{***}	16.015^{***}
Treatment (T)	1	16.378^{***}	13.628^{***}	16.292^{***}	0.816^{*}	5.167^{**}
Sampling time (S)	3	2.717^{***}	5.958^{***}	3.642^{***}	0.198^{ns}	0.298^{ns}
cv. × T	1	0.097^{ns}	0.124^{ns}	3.616^{***}	3.432^{***}	3.555^{**}
cv. × S	3	0.668^{ns}	1.904^{***}	0.254^{*}	1.662^{***}	0.311^{ns}
T × S	3	2.402^{***}	1.515^{***}	2.110^{***}	0.247^{ns}	0.976^{ns}
cv. × T × S	3	0.102^{ns}	0.068^{ns}	0.486^{ns}	0.530^{**}	0.409^{ns}
Error	32	0.302	0.169	0.131	0.111	0.440
CV%		18.9	14.4	18.1	11.1	19.1

- SOV, Source of variation. df, Degrees of freedom. CV, Coefficient of variation.
- ^{ns} Non-significant (P > 0.05). *, **, *** Significant at P < 0.05, P < 0.01, and P < 0.001, respectively.

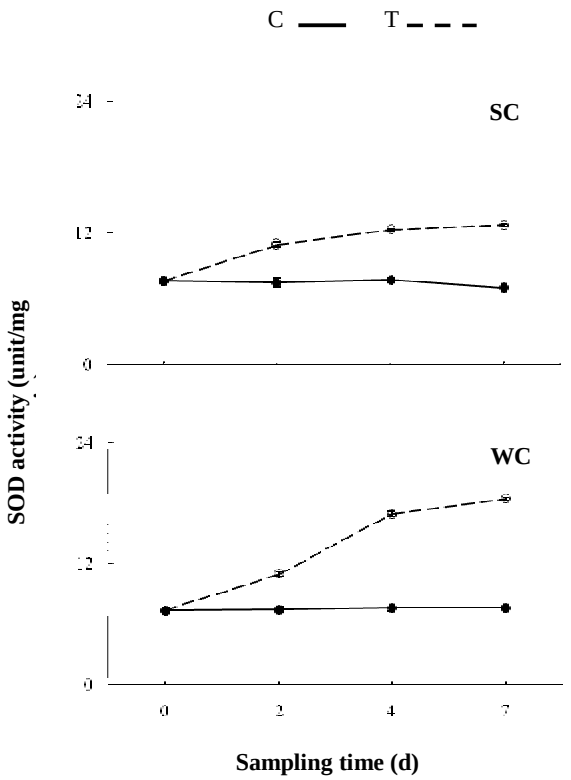

Figure 1. Changes in SOD activity in leaves of Rgs003 (SC= spring canola) and Okapi (WC = winter canola) grown either at 22/16°C (C = control) or at 10/3°C (T = cold treatment) over sampling times. Values are means (*n* = 3) ± SE, but where bars are absent, the variation about the mean was less than the diameter of the symbol.

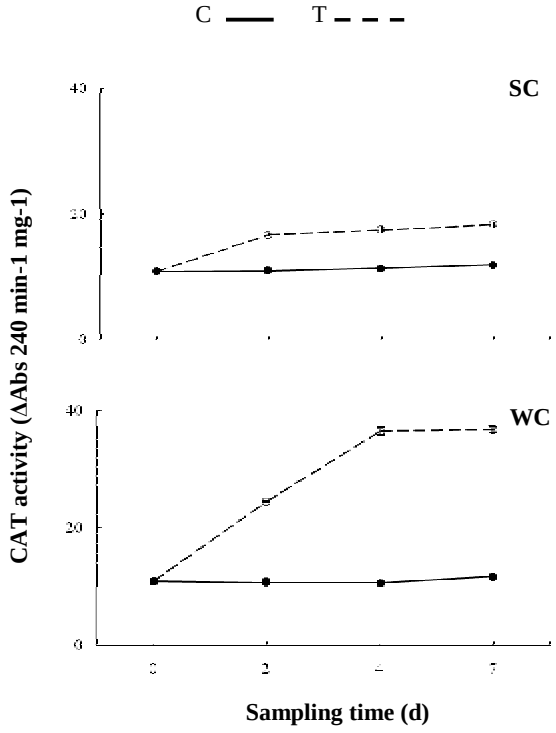

Figure 2. Changes in CAT activity in leaves of Rgs003 (SC= spring canola) and Okapi (WC = winter canola) grown either at 22/16°C (C = control) or at 10/3°C (T = cold treatment) over sampling times. Values are means (*n* = 3) ± SE, but where bars are absent, the variation about the mean was less than the diameter of the symbol.

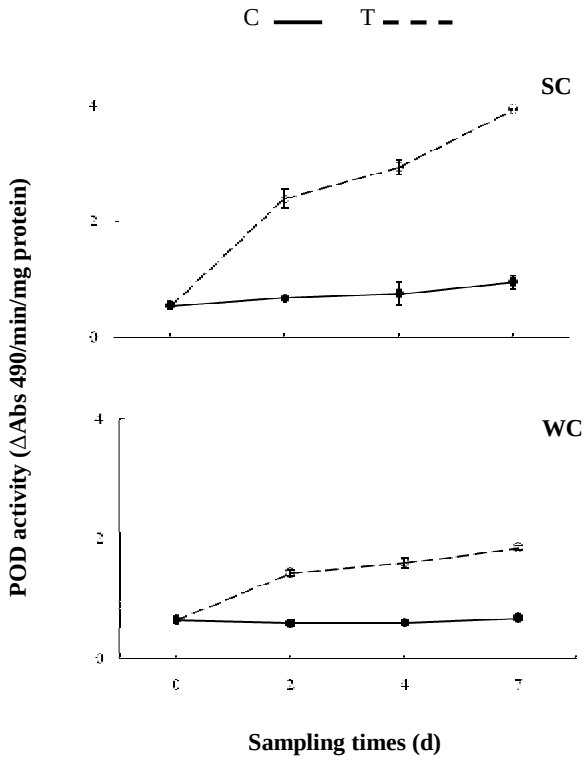

Figure 3. Changes in POD activity in leaves of Rgs003 (SC= spring canola) and Okapi (WC = winter canola) grown either at 22/16°C (C = control) or at 10/3°C (T = cold treatment) over sampling times. Values are means (*n* = 3) ± SE, but where bars are absent, the variation about the mean was less than the diameter of the symbol.

in both canola cultivars (Table 1). On 7th day of cold exposure, the activity of CAT enhanced by 3.1-fold (P<0.001, Figure 2) cold-treated leaves of WC compared with those of the control: to a very less extent, it was true about SC cultivar at this time (1.5-fold increase, P<0.001, Figure 2). CAT higher activity, against what is accompanied by enhancement of other antioxidant enzymes activity, does not reduce plants store of energy because among several ROS detoxifying enzymes, CAT requires no supply of reducing equivalents (Ozkur *et al*. 2009). It is an important advantage especially in the stress conditions where photosynthesis rate reduces and the plant's reservation of energy gets poor. Thus, there is a strong correlation between CAT activity and stress tolerance in plants (Juhnke 1996). High activity of CAT indicated drought tolerance in some of the canola cultivars (Tohidi-Moghaddam *et al*. 2009; Omidi 2010) and cold treatment enhanced CAT activity in some wheat cultivars (Javadian *et al.* 2010). On the other hand, as mentioned above, superoxide radical is turned to H_2O_2 and O_2 by SOD function then POD, CAT and APX detoxify H_2O_2 under different antioxidant cycles (Ozkur *et al*. 2009). Since CAT has the highest affinity to H_2O_2, higher corporation between SOD and CAT leads to more tolerance as the drought tolerant canola showed more SOD and CAT correlation under salt stress

(Rajabi *et al.* 2012). Our present report verified that Okapi as a winter cold tolerant cultivar showed higher level of this corporation under cold treatment (Chelikani *et al.* 2004). Similar results were reported for other crops, e.g. wheat, where the winter type showed higher CAT activity than the spring type under cold treatment (Baek and Skinner 2003) and soybean plants under cold stress (Balestrasse *et al.* 2010). Similar to the above-mentioned enzymes (SOD and CAT), no remarkable changes happened in the activity of POD during the experimental sampling times in the control seedlings of both canola cultivars but, cold treatment caused enhanced activity in cold-induced leaves of both canola cultivars compared with the control. In contrast with SOD and CAT enzymes, the POD activity enhanced more in cold-induced leaves of Rgs003 (Figure 3, SC) as a spring cold sensitive canola cultivar compared with those of Okapi (Figure 3, WC) as a cold tolerant cultivar. In other words, on 7^{th} day of cold treatment, the activity of POD increased by 4.2-fold ($P<0.001$) in SC cold-treated leaves compared with those of the controls: to a lesser extent, it was detectable in WC cultivar at this time (2.7-fold increase, $P<0.001$). Similarly, it has been previously shown that POD activity in maize, tobacco, *Cucumis sativus*, tomato and canola increased under cold treatment (Prasad 1996; Parvanova *et al.* 2004; Javadian *et al*. 2010; Zhang *et al.* 2011; Duan *et al.* 2012). This does not mean the more efficiency of antioxidant system in Rgs003 cultivar because of two reasons. First, POD had less affinity to H_2O_2 in comparison with CAT and second, POD depended on energetic molecules of photosynthesis system for its function. Therefore, less activity of CAT and more POD activity led to more energy consumption and surely, less efficiency of H_2O_2 detoxification as what happened in cold-induced leaves of Rgs003 (Mittler 2002). Moreover, increased activity of POD usually causes cross links in the cell wall, more wall stiffening and growth limitation of the plants (Duan 2012). Cold treatment caused no noticeable changes in APX activity in SC (Figure 4) whereas, APX activity enhanced by 1.7-fold (Figure 4) in cold-treated leaves of WC as a winter cultivar on the second day of cold exposure compared with those of the controls: this response lessened to 1.2-fold increase on subsequent days of 4 and 7 ($P<0.001$, Figure 4). Based on different affinities of APX (μM range) and CAT (mM range) for H_2O_2, APX is more likely responsible for the fine modulation of ROS in signaling pathway whereas, CAT might be responsible for removal of excess ROS during stress (Lopez-Huertas 2000; Gill 2010). Sudden enhancement of APX activity in cold- treated leaves of Okapi at the second day of cold treatment could be the evidence showing APX interference at cold signal transduction. On the other hand, cold enhancement of this enzyme could mean the higher efficiency of H_2O-H_2O cycle and ascorbate-glutathione cycle as perfect antioxidant mechanisms in Okapi as a cold tolerant cultivar compared with Rgs003 as a cold sensitive cultivar of canola (Mittler 2002). Similar results were also reported under drought stress in Okapi compared to RGS (Omidi 2010).

Effect of cold on lipid peroxidation

Lipid peroxidation is a process caused by ROS and leads to sever cell membrane damages. Cell membrane stability has strong correlation with abiotic stresses. MDA is an important index of lipid peroxidation rate and oxidative stresses damages (Uemura *et al*. 2006; Kazemi Shahandashti 2012). Cold treatment caused increased MDA amounts with time in a linear form in cold-induced leaves of SC (Figure 5) compared with the control. On day 7 of cold exposure, the amount of MDA was doubled ($P<0.001$) in SC cold-treated leaves compared with the controls. In contrast to SC, cold treatment caused no changes in the amount of MDA in WC seedlings (Figure 5). No notable changes in the amount of MDA in Okapi and the increment of it in Rgs003 during cold treatment, indicate that Okapi had higher ROS scavenging mechanism efficiency as a winter and cold tolerant cultivar, compared with Rgs003 as a spring and cold sensitive canola cultivar. In conclusion, in this assay, we showed more cold tolerance in Okapi as a winter and cold tolerant cultivar because of more activities of three important antioxidant enzymes *i.e*., SOD, CAT and APX and less consumption of energetic molecules.

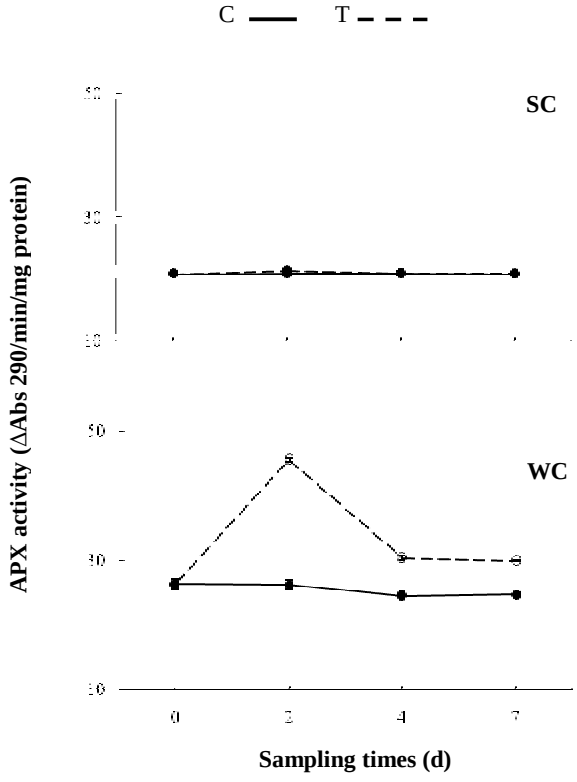

Figure 4. Changes in APX activity in leaves of Rgs003 (SC= spring canola) and Okapi (WC = winter canola) grown either at 22/16°C (C = control) or at 10/3°C (T = cold treatment) over sampling times. Values are means (*n* = 3) ± SE, but where bars are absent, the variation about the mean was less than the diameter of the symbol.

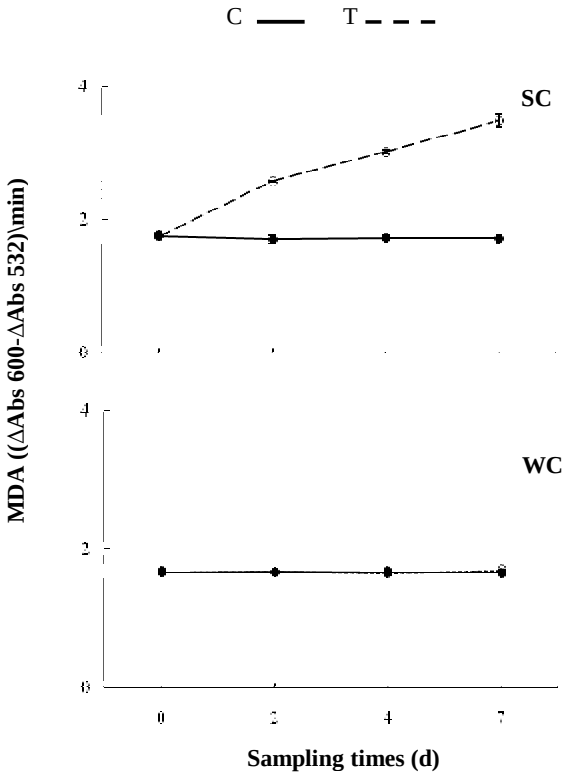

Figure 5. Changes in MDA in leaves of Rgs003 (SC= spring canola) and Okapi (WC = winter canola) grown either at 22/16°C (C= control) or at 10/3°C (T = cold treatment) over sampling times. Values are means (*n* = 3) ± SE, but where bars are absent, the variation about the mean was less than the diameter of the symbol.

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