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## Aerials and Roots Vegetative Tissues Differ in Guaiacol Peroxidases Responses to Cold Stress in Two Contrasting *Medicago ciliaris* Populations

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**Abstract** Effect of cold on antioxidant responses in aerial (shoots and leaves) and roots tissues of two contrasting accessions of *M. ciliaris* Krockers (Cil 126, tolerant and Cil 123, sensible) were investigated. Ten-day-old grown seedlings were subjected at different periods of cold acclimation (4°C) 2, 4, and 6 days. Peroxidases (POD) (EC 1.11.1.7) activities and isoenzymes expression of plantlets treated and control (23°C) of the aerial (shoot+leaves) and root vegetative tissues were carried. Global peroxidase activity under low temperature stress was higher in tolerant ecotype than sensible one. The same trend was also observed at expressed isoenzymes. On the other hand, whether in tolerant or sensitive, activity and isoenzymes peroxidase was more pronounced at the roots than in aerials tissues system.

**Keywords** Peroxidase activity; Isoenzymes; Cold stress; *M. ciliaris*; PAGE electrophoresis

### Introduction

Cold stress is one of the major factors limiting agricultural productivity, consequently the plants evolved a numerous adaptive responses to cope with environmental stresses. Agricultural yield losses due to abiotic environmental stresses are obvious and have been well-documented (Cramer et al., 2011). Abiotic and biotic stress often causes a series of morphological, physiological, biochemical and molecular changes that unfavourably affect plant growth, development and productivity. Drought, salinity, extreme temperature (cold and heat) and oxidative stress are often interrelated; these conditions singularly or in combination induce cellular damage (Arun et al., 2011; Prince et al., 2013; Morina et al., 2015). Ghosh and Xu (2014) reviewed abiotic stress responses in plants and reported that these responses occur in various organs especially at roots tissues. Under normal growth condition, root absorbs water and nutrients from the soil and supplies them throughout the plant body, ensuring a cellular homeostasis balance. However, this cellular homeostasis altered during the stress period when roots tissues are forced to adopt several structural and functional modifications. Under cold stress a significant amount of reactive oxygen species (ROS) is produced, such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and other dangerous derivatives oxygen, which cause progressive oxidative damage and as consequence cell death (Mohammadian et al., 2012). During cold acclimation, increases of enzymatic changes were associated with cold tolerance by a significant capture of these harmful molecules. It is often assumed that in many species, plants actively produce ROS as signalling molecules to control processes such as programmed cell death (Parent et al., 2008), abiotic stress responses, for example, in Barley (Baek et al., 2000), in rice (Kim and Thomas, 2011), in maize (*Zeamays* L.) (Lukatkin, 2002), pathogen defense, in Pepper (Wang et al., 2013). Higher plants contain numerous enzymatic and non-enzymatic reactive oxygen intermediate scavengers and antioxidants, both water and lipid soluble, localized in different cellular compartments (Mittler, 2002; Thakur and Nayyar, 2013). Antioxidant enzyme activities in plants are accepted as a good indicator of tolerance under stress conditions (Öklen et al., 2008).

Lee et al., (2009) argument that roots proteins increases highly under cold stress than in the leaves in rice. On the other hand, Cavalcanti et al., (2007) investigating the effect of salt stress in cowpea (*Vigna unguiculata*) plants, concluded that roots and leaves display distinct mechanisms of response during salt stress and recovery.

Radić et al. (2006) analyzing the peroxidase activity in leaves and roots under salt stress, highlighted that POD activity of salt-stressed plants changed with respect to different saline treatments and plant organs and this activity was mainly due to the *de novo* synthesis of the enzyme in shoot and root.

Lee and Lee (2000) analyzing the changes of antioxidant enzyme isoforms against chilling stress in the leaves of cucumber (*Cucumis sativus* L.) found five APX isoforms were presented in the leaves of cucumber. Furthermore, these authors showed that intensities of APX-4 and APX-5 were enhanced by chilling stress, whereas that of APX-3 was significantly increased in the post stress periods after chilling stress. Wang et al., (2009) suggested that peroxidase (POD) activity in normal conditions in two alfalfa cultivars was higher in root than in shoots. However, POD activities in shoot and root tissues of both cultivars showed similar level under chilling stress.

Keshavkant and Naithani (2001) reported that aerial parts of the chilling sensitive young sal (*Shorea robusta*) seedlings showed overproduction of reactive oxygen species. To gain in a clear understanding of the reactions exhibited by annuals *Medicago* plants under different duration of cold stress; we conducted a study with enzyme activity (peroxidase) and peroxidase isoenzymatic pattern as biochemical markers in response to cold stress in the aerial and roots tissues. These results may help in the development of a new variety of stress tolerant *Medicago* plants.

## 1 Results and Discussion

The biochemical changes observed in our study during cold stress in both vegetative tissues has been demonstrated in many species and under many stresses. Sánchez-Calderón et al., (2013) showed that in root vegetative system has to grow in media where the biotic and abiotic components are distributed heterogeneous. As soon as the root makes contact with the soil must sense and integrate biotic and abiotic cues in order to adjust their genetic program of post-embryonic root development. Ghosh and Xu (2014) reported that proteomic analyses of plant roots under various abiotic stress conditions revealed important information on proteins involved in the abiotic stress response. This leads to the identification of molecular and cellular mechanisms that are specific to certain abiotic stress or shared between two or more abiotic stress conditions. On the other hand, these authors highlighted that higher abundance of ROS scavengers was detected in roots under drought, high salinity and cold stress and can be looked upon as a preventive measure against oxidative damage caused due to high ROS levels.

To gain a better knowledge of the chilling response, Lee et al., (2009) suggested that it is imperative to analyze the tissue-specific proteome patterns under chilling stress. Prasad (1996) investigated the mechanisms involved in chilling injury or tolerance in developing maize seedlings subjected to low temperature, noted that peroxidase activities in acclimated plants increased gradually with 14 to 120 % with age and remained slightly higher during recovery in 2- to 4- days- old seedlings. Cavalcanti et al., (2007) comparing some antioxidative response during short-term salt stress, spotlighted that roots and leaves display distinct mechanisms of response during salt stress and recovery. However, these responses and/or the oxidative damage caused by oxygen species were not related with the growth reduction.

### 1.1 Changes in the activity of POD

Chilling tolerance or sensitivity in plants is well correlated with inherent antioxidant responses. Tolerant plant species generally have a better capacity to protect themselves from chilling-induced oxidative stress, via the enhancement of antioxidant enzyme activity. In plants, elevated ROS without an efficient scavenging system may lead to high injury index and finally death of the whole plant (Azzeme et al., 2016). These authors noted that in response to different levels of drought in the root and leaf tissues in oil palm plants the guaiacol POD activity in leaves was found to increase sharply under mild stress but dropped under moderate and further reduced under severe drought conditions even though the levels were still higher than that for the control. In the roots, the guaiacol POD activity significantly increased under mild and further increased moderate and severe stress conditions. Liu et al., (2013) have observed an enhancement in activities peroxidase synthesis pathway during cold acclimation. Yadegari et al., (2007) showed in soybean a significant changes in guaiacol peroxidase activity

between cold acclimated and non-acclimated leaves 24 h after acclimation.

In our study, we observed increasing of patterns of antioxidant enzyme activities in both aerial and root system under cold stress comparing with control in tolerant and sensible ecotypes. Whereas, under these activities were more pronounced in root than in aerial (leaves and shoots) tissues (Figure 1). The result was in agreement with the findings of Wang et al., (2009). Furthermore, Kuk et al. (2003) obtain similar results, where they explain that the antioxidant activity in rice is higher in the roots than in the leaves. Under cold stress the activities was better in root than in aerial tissues. (Figure 1A; Figure 1B). In tolerant ecotype (Cil 126), the guaiacol POD activity in the aerial tissues (leaves and shoots) (Figure 1A) was found increased in T4 treatment ( $0.174 \times 10^{-3} \mu\text{mole/min/g FM}$ ) compared with control. This level of activity was substantially decreased at the 6 days of cold treatment ( $1.1 \times 10^{-3} \mu\text{moles/min/g FM}$ ) compared with control ( $1.17 \times 10^{-3} \mu\text{moles/min/g FM}$ ). Nevertheless, dropped sharply at the beginning of cold treatment (T2) ( $0.080 \times 10^{-3} \mu\text{moles/min/g FM}$ ) compared with control ( $0.161 \times 10^{-3} \mu\text{moles/min/g FM}$ ). In the roots, the guaiacol POD activity (Figure 1B) showed accounted for a steady increase in treated plantlets compared to the control plants according to the duration of treatment. Furthermore, POD activity at T6 cold treatment ( $1.54 \times 10^{-3} \mu\text{moles/min/g FM}$ ), showed a significant increase ( $P \leq 0.05$ ) (data not showed) compared to the control ( $1.19 \times 10^{-3} \mu\text{moles/min/g FM}$ ).

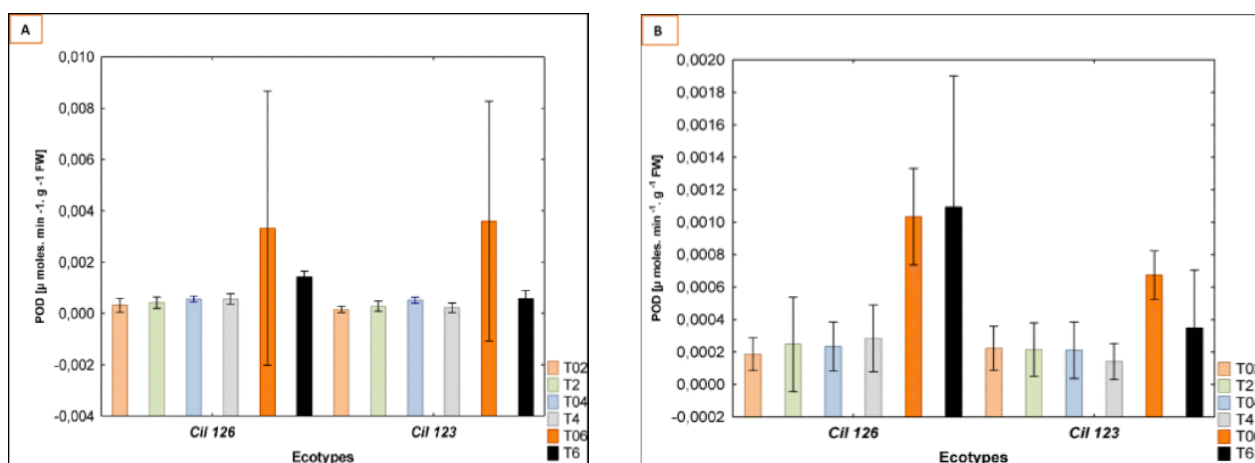


Figure 1A: Aerial vegetative tissues peroxidase activity in *M. ciliaris* Krockers.ecotypes (Cil 126 and Cil 123) seedlings untreated (controls): (T02, T04, and T06) and treated with different durations (T2, T4, and T6) under cold stress. Data are shown as mean  $\pm$  SD of three independent measurements

Figure 1B: Root vegetative tissues peroxidase activity in *M. ciliaris* Krockers.ecotypes (Cil 126 and Cil 123) seedlings untreated (controls): (T02, T04, and T06) and treated with different durations (T2, T4, and T6) under cold stress. Data are shown as mean  $\pm$  SD of three independent measurements

In sensible one (Cil 123), the same tendency of guaiacol POD activity was observed but with less intensity compared to the tolerant ecotype. It is more cleared that the guaiacol peroxidases activity was greater in root tissue than in aerial tissues in two contrasting ecotypes (Figure 1A; Figure 1B). Kuk et al., (2003) obtain similar results, where they explain that the antioxidant activity in rice is higher in the roots than in the aerial parts (buds). On another side, Okuda et al., (1991) stippled that  $\text{H}_2\text{O}_2$  content increase sharply in wheat leaves under chilling stress. Lee et al., (2009) highlighted the same trend. These authors argue that the roots are most affected by cold stress and energy metabolism pathway in rice roots is more affected by the cold stress. Cold stress causes inhibition of photosynthesis in leaves and influence on various metabolic reactions that are at the origin of the overproduction of  $\text{H}_2\text{O}_2$  (Larkindale et al., 2005). Regarding Gasparet al., (1992) and Kukavicaet al., (2007), these authors noted that the production of peroxidases was actively involved in root growth.

## 1.2 Effect of cold stress on peroxidase isoforms pattern

Guaiacol peroxidases are known to utilize different substrates to metabolize  $\text{H}_2\text{O}_2$ . When guaiacol was used as a

substrate, peroxidase activities were enhanced in chilling stressed-plant as compared with control plant (Lee and Lee, 2000). Comparing the peroxidase profiles between control plants and those treated under cold stress using native-page, the pattern of isoenzyme showed that cold treatment induced a high peroxidase activity. This activity increase slightly in roots tissues (Figure 2, A) than in aerial (leave and shoot) tissues (Figure 2, B) in both ecotypes. The peroxidase activities were more pronounced when cold stress is maintained in the time. This trend was observed in tolerant accession Cil 126 when plantlets were subjected at 6 days of stress. In contrast, in sensible accession Cil 123 peroxidase activities were slightly maintained (Figure 3: A and B). The same results were observed in chickpea (*Cicer orientum* L.) (Nazari et al., 2012). It was found that the number of bands (named POD-Rfs) was higher (five) in tolerant (Cil 126) (Figure 2) than in sensible (Cil 123) (Figure 3) with only four bands (POD 1 to POD 4). Moreover, results revealed that under cold treatment the intensity of bands were more pronounced in roots (Figure 2, A and Figure 3, A) than in aerial tissues (Figure 2, B and Figure 3, B). As showed in Figure 2A the intensity of POD 5-Rf\_0.89 of Cil 126 and in Figure 3A POD 4-Rf\_0.69 of Cil 123 in root was increased in cold treated plants in comparison of aerial tissues in both tolerant and sensible accession.

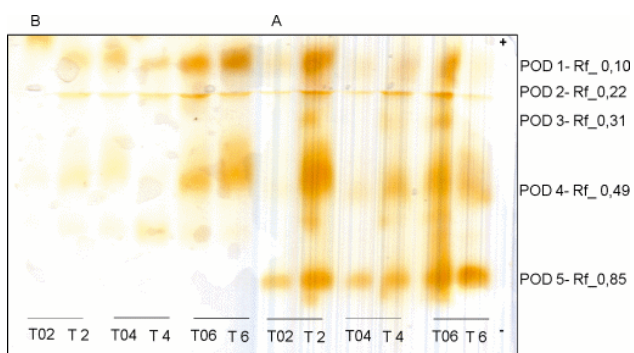


Figure 2 Peroxidase isoenzyme patterns in *M. ciliaris* Krockers. (Cil 126) seedlings untreated (controls): (T02, T04, and T06) and treated with different durations (T2, T4, and T6) under cold stress

Notes: A: Root vegetative tissues and B: Aerial vegetative

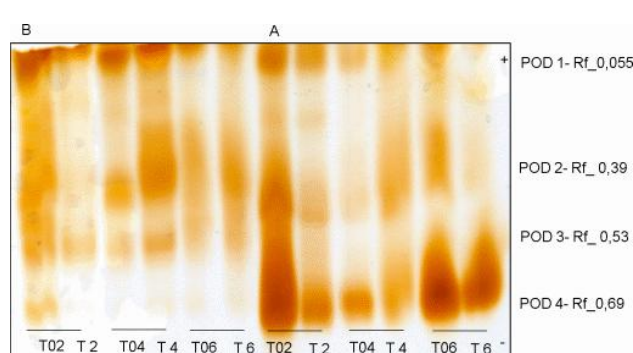


Figure 3 Peroxidase isoenzyme patterns in *M. ciliaris* Krockers. (Cil 123) seedlings untreated (controls): (T02, T04, and T06) and treated with different durations (T2, T4, and T6) under cold stress

Notes: A: Root vegetative tissues and B: Aerial vegetative

Mohammadian et al., (2012) showed that in tissues of three cultivars of lemon (*Citrus unshiu*, *Citrus sinensis* and *Citrus lemon*) the intensity of peroxidase bands is low in the sensible cultivars and is more important with 3 intense bands in both tolerant cultivars (lemon *Citrus sinensis* and *Citrus lemon*) under a cold treatment. Furthermore, Lee and Lee (2000) have observed this tendency. These authors, analyzing the changes of antioxidant enzyme isoforms against chilling stress in the leaves of cucumber (*Cucumis sativus* L.), found five APX isoforms were presented in the leaves of cucumber. Furthermore, these authors showed that intensities of APX-4 and -5 were enhanced by chilling stress, whereas that of APX-3 was significantly increased in the post stress periods after chilling stress. On the other hand, Azzeme et al., (2016) noted that POD activity increased compared to control under different severity of drought in both tissues (leaves and roots) which showed that POD is an essential enzyme in scavenging  $H_2O_2$  in the oil palm seedlings. Mirzaee et al., (2013) reported an initial increase and later decrease in antioxidant activities in leaves and roots tissues of *Brassica napus* L. compared to control plants with the increasing duration of stress indicating the unbalance of the active oxygen metabolism system. The high accumulation of active oxygen was able to initiate and accelerate lipid peroxidation. Nevertheless, when the levels of the active oxygen exceeded the ability of antioxidant system to cope with them, damage to cellular components occurred (Cao et al., 2011).

## 2 Conclusion

Based on obtained results, it could be concluded that activities of peroxidase had different change trends with tolerant and sensible accession. The enhanced of scavenging ability for  $H_2O_2$  in tolerant was better than in sensible accession. Two accessions Cil 126 and Cil 123 has potentially better antioxidant potential in roots tissues than in aerial tissues. Cold adversely affects the plant metabolism in *Medicago* populations seedlings by affecting antioxidant enzyme activities in leaves and roots tissues. Thus, this work can be benefited as potential targets for enhancement of stress tolerance in *Medicago* species under different durations, through genetic of mRNAs transcripts engineering and breeding programmes to develop cold tolerance local annuals *Medicago* cultivars.

## 3 Materials and Methods

The study was carried on two annual *Medicago ciliaris* Krockers (Cil 126, tolerant and Cil 123, sensible) (Table 1). Ten seeds for each accession were germinated after scarification and disinfected by dipping in 70% (v/v) ethanol, at temperature room in Petri dishes containing universal compost imbibed with distiller water. At three days growth stage, seedlings were divided into two lots. Cold treated lot at 4 °C for three durations 2, 4 and 6 days (T2, T4 and T6) and control lot kept at 23 °C (T02, T04 and T06). The experimental layout was a completely randomized block design with 3 replications.

Table 1 Accessions analyzed for peroxidase antioxidant under cold stress with their origin and ecological description

Species	Accessions	Origin	Latitude	Longitude
<i>M. ciliaris</i> Krockers.	Cil 123 (S)	Algeria	36°46'02"N	8° 18' 9.57" E
	Cil 126 (T)	Algeria	36° 28' 0" N	7° 26' 0" E

Notes: S: sensible, T: tolerant

Aerial (shoot and leaves) and root tissues from control and treated plants (Figure 4) were homogenized in homogenization buffer (10 mM Tris-KCl, pH 6.8, 10% (w/v) saccharose and 1 mM PMSF). Tissues were frozen in liquid nitrogen and ground in mortar on ice using homogenization buffer.



Figure 4 Aerial (shoot and leaves) and root tissues from control and treated plantlets

Peroxidase activity (EC 1.11.1.7) (POD) was assayed in reaction solution (3 ml) containing 0.2 M sodium acetate buffer pH 4.6, 1% o-dianizidine, 10%  $H_2O_2$  (Mac Adam et al., 1992) modified. The reaction was started by adding 10 µl crude extract, and the enzyme activity is monitored for every 15 seconds for 3 minutes using a



spectrophotometer at 460 nm as o-dianizidine oxidation, with 3 replications. Enzyme activity was expressed as  $\mu$ mole of o-dianizidine oxidation. FM/mg of fresh matter (FM).

The aerial (shoot and leaves) and root tissues peroxidase pattern were resolved by native in 10% separating polyacrylamide gelelectrophoresis on vertical slab gel (Hoffer, USA) using the procedure of Laemmli (1970). Equal amounts of proteins (15  $\mu$ l) were loaded on to each lane. The gel electrophoresis experiment was repeated three times. To determine the pattern of peroxidase isoforms gels were staining and visualized by immersing the gels in 100 ml 0.2 M acetate buffer pH 4.6 added with 1% o-dianizidine (Sigma), dissolved in 2 ml 95% ethanol, and 200  $\mu$ l 3% H<sub>2</sub>O<sub>2</sub> at room temperature until the brown color appeared. Scanned Isozymes profiles gels were analyzed by Software GELANALYZER (Istvan Lazar Hungary Copyright 2010) to determine Rf bands.

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#### Authors' contributions

YN conceived of the study, design it has and performed the statistical analysis and analysis results. BA and CC carried out the research as part of their MSc project. F-LFZ have been involved in the revising it critically for intellectual content. All authors have participated sufficiently in the work and all authors read and approved the final manuscript.

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