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Alleviation of waterlogging damage by foliar application of nitrogen compounds and tricyclazole in canola

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Abstract

Waterlogging stress induces harmful physiological changes which restrict canola growth. The aim of this investigation was to evaluate the effects of foliar applications of nitrogen compounds (urea, calcium nitrate and potassium nitrate) and tricyclazole at different growth times on physiological responses in canola plants (*Brassica napus* L. cv. Hayola 401) subjected to waterlogging stress. Plants in the 5-leaf growth stage were exposed to waterlogging conditions for two weeks. Results of waterlogged control compared to non-waterlogged control demonstrated that waterlogging stress significantly decreased the activity of superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6) and peroxidase (POX; EC 1.11.1.7), while lipid peroxidation and ethylene production in the leaves were increased by the waterlogging stress. Furthermore, dry weight and length of shoots and roots were reduced by waterlogging stress. These adverse effects of waterlogging stress were alleviated by foliar application of all tested compounds; once (before the stress) or twice (before and after the stress). Twice foliar applications did not have any significant superiority to once foliar spray. It was concluded that, among the different treatments, the foliar spray of calcium nitrate, potassium nitrate and tricyclazole before the waterlogging stress were more beneficial and cost efficient than the twice application and is advisable to alleviate the waterlogging damage in canola.

Keywords: Antioxidants; Calcium nitrate; Ethylene; Malondialdehyde; Oxidative stress; Potassium nitrate; Tricyclazole; Urea; Waterlogging.
Abbreviations: BSA-bovine serum albumin; CAT-catalase; FSAW-foliar spray after waterlogging; FSBW-foliar spray before waterlogging; FSBAW-foliar spray before and after waterlogging; MDA-malondialdehyde; NBT-nitrobluetetrazolium; POX-peroxidase; ROS-reactive oxygen species; SOD-superoxide dismutase.

Introduction

Canola (*Brassica napus* L.) is an important oilseed crop and is considered as a good source of edible oil. Its cultivation is increasingly being used, as a rotation crop following rice. Waterlogging is a major constraint for production and productivity of many crops (Leul and Zhou 1998). The lack of O_2 due to waterlogging may limit the plant growth due to the alterations in metabolism and nutrient uptake of plants, leading to the creation of reactive oxygen species (Drew 1992). Plants may respond to waterlogged conditions by altering their hormone balance (Grichko and Glick 2001) and the abnormal growth can occur due to overproduction of ethylene in the shoots (Saleem et al., 2007). Nutrient deficiency is the major cause of poor plant growth in waterlogged soil (Steffens et al., 2005). Waterlogging causes a significant decrease in nitrogen content in plants due to reduced root activity. Yellowing of leaves due to loss of chlorophyll from leaves of waterlogged plants is attributed to nitrogen deficiency (Rao et al., 2002). Nitrogen deficiency may be induced by the low redox potential in waterlogged soil that promotes denitrification of NO_3^-. Under anaerobic conditions, root metabolism and root growth are inhibited, as the lack of O_2 affects the energy status of the plant (Drew 1992; Voesenek et al., 2003). Gutierrez Boem et al. (1996) reported that waterlogging resulted in a decrease of nitrogen (N), phosphorus (P), potassium (K) and calcium (Ca) uptake by canola. A strategic use of fertilizers prior to waterlogging stress may alleviate nutrient deficiency. While the deleterious effects of waterlogging have often been partially counteracted by the addition of fertilizers (mainly nitrogen containing) to pots or in the fields, they did not fully overcome them, perhaps due to the reduced ability of roots to absorb nutrients. Foliar sprays of nitrogen fertilizer could alleviate canola damage caused by waterlogging by retarding chlorophyll and nitrogen degradation and increasing superoxide dismutase and catalase activities (Zhou et al., 1997). Another study showed that the foliar spray of nitrogen ameliorated the effects of waterlogging by restoring chlorophyll concentration in the leaves of corn plants (Rao et al., 2002). Waterlogging damages may be alleviated by applying a suitable plant growth regulator at appropriate growth stage (Zhou et al., 1997). Triazoles have both fungitoxic and plant-growth regulatory effects. They can also protect plants against various stresses. Therefore, the triazoles have been characterized as plant multi-protectants (Leul and Zhou, 1998). The results of recent studies indicated that paclobutrazol, a closely related triazole, and mixtalol could alleviate waterlogging damage in canola and sweet potato plants (Zhou et al., 1997; Lin et al., 2006). Uniconazole, another member of the triazoles, increases the activity of antioxidant enzymes and chlorophyll content in

canola (Leul and Zhou 1999). Tricyclazole [5-Methyl-1,2,4-triazole(3,4-b) benzothiazole] is a triazole fungicide often applied by spraying to treat rice blast disease (Sancho et al., 2009), but its potential to alleviate the damages in plants subjected to waterlogging stress must be investigated. However, no research has yet been done to compare the effects of foliar applications of various nitrogen compounds and tricyclazole on waterlogged-canola. The objective of this work was to compare the effects of foliar applications of urea, calcium nitrate, potassium nitrate and tricyclazole on responses of canola plants under waterlogging stress.

Results

Antioxidant activity

The experimental treatments had a significant effect ($p < 0.01$) on the catalase (CAT), peroxidase (POX) and superoxide dismutase (SOD) activities in canola leaves (Table 1). Waterlogging stress caused a significant decrease in CAT, POX and SOD activities in canola leaves compared with the non-waterlogged (NWL) control (Fig 1 a, b and c). The application of all compounds as foliar spray before waterlogging (FSBW) significantly increased CAT (except for urea), POX and SOD activities compared to the waterlogged control (WL). However, there was no significant difference between FSBW of $Ca(NO_3)_2$ and NWL control. The foliar spray after waterlogging (FSAW) of all compounds caused a significant increase in CAT, POX (except for urea and tricyclazole) and SOD activity compared to the WL control. The superior results caused either by $Ca(NO_3)_2$ or KNO_3. The foliar spray before and after waterlogging (FSBAW) of all compounds significantly increased the CAT, POX and SOD activity compared to the WL control, while calcium nitrate and potassium nitrate had superior results.

Lipid peroxidation and ethylene production

The effects of experimental treatments on the amount of malondialdehyde (MDA) and ethylene production in canola leaves were statistically significant ($p < 0.01$), as illustrated in Table 1 and 2. The amount of MDA (Fig 1 d) and ethylene production (Fig 2 a) significantly increased in canola leaves from waterlogging stress. Foliar spray of all compounds in each time of application that was tested significantly decreased MDA and ethylene compared to the WL control. FSBAW or FSBW of $Ca(NO_3)_2$, KNO_3 and tricyclazole caused a lower MDA or ethylene production in leaves.

Dry weight and length of root and shoot

The experimental treatments had a significant effect ($p < 0.01$) on dry weight and length of root and shoots (Table 2). Root and shoot dry weight significantly decreased following waterlogging stress (Fig 2 b and c). The FSBW and FSBAW of all compounds significantly increased dry weight of root and shoot (except for FSBW of urea) compared with the WL control, although the lower increase was obtained by foliar application of urea. Length of root and shoot were also significantly decreased due to the waterlogging stress (Fig 2 d and e). The foliar application of all compounds resulted in a significantly longer root length, though the effects of FSBW or FSBAW were greater. The effect of urea on the increase in root length was less pronounced than that of the other compounds. The effects of FSBW and FSBAW of all compounds were significant on the increase in shoot length compared with the WL control, except for FSBW of urea which had no significant effect. The effect of FSAW of all compounds was not significant on shoot length compared with the WL control.

Discussion

The results of this experiment provide new clarification of the mechanism that underlies the ability of nitrogen compounds and tricyclazole to help plants tolerate waterlogging stress. It is known that leaf senescence is regulated by several factors including genetic, hormonal and environmental signals or stresses (Liu et al., 2010). Thus, leaf senescence of canola seedlings in this experiment is probably due to the effect of waterlogging on these factors. The application of all compounds significantly alleviated the growth-inhibiting effects of waterlogging stress. Waterlogging stress induced leaf senescence evident in the higher MDA content and the overproduction of ethylene, an inhibitor of plant growth (Bleecker and Kende 2000). Leul and Zhou (1998) observed an increase in the level of ethylene production in canola leaves as a result of waterlogging stress. The reduction in ethylene and MDA content (diminution in leaf senescence) due to the foliar applications of treatments is evidence in support of this hypothesis. In the current study, the application of nitrogen compounds significantly alleviated the negative effects of waterlogging stress. Nitrogen deficiency, as the primary detrimental effect caused by waterlogging, stimulates lipid peroxidation and pigment loss as well as protein degradation that leads to the inhibition of photosynthetic capacity and ultimately results in leaf senescence (Casano et al., 1994). Nitrogen also has a role in the synthesis of cytokinins (CKs), which is important for chlorophyll synthesis and protection against leaf senescence (Wingler et al., 1998; Argueso et al., 2009). Calcium (Ca) has a very important role, not only for cell wall and membrane stabilization, but is also involved in the regulation of specific plant responses to environmental stresses (Braam et al., 1996). Ca^{2+} inhibits or slows leaf tissue senescence through cross-linking pectates and cementing cell walls. It also has a positive effect on SOD activity (Schmitz-Eiberger et al., 2002). Potassium (K) plays an important function in photosynthetic pigments and photosynthetic capacity via effects on the activities of several photosynthetic enzymes (Ashraf et al., 2011). Cong et al. (2009) have also shown that the application of K along with N and P significantly improved tolerance to waterlogged conditions. In this study, antioxidant enzyme activity and MDA content indicated that oxidative stress is a significant consequence of waterlogging stress in canola. Decreased shoot and root growth could be as a result of oxidative stress induced by waterlogged conditions. The foliar applied treatments could significantly reduce oxidative stress as evident by enhanced antioxidant enzyme activity and consequently diminished MDA content. MDA content is often an indicator of lipid peroxidation in plant tissue that results from oxidative stress induced by various abiotic stresses (Tang et al., 2010). Waterlogging leads to oxidative stress in plants through an increased production of reactive oxygen species (ROS) which trigger a series of deleterious processes, such as lipid peroxidation, degradation of proteins and DNA damage in the cell (Scandalios 1993). Resistance to waterlogging stress may depend, at least in part, on increased activity of antioxidant enzyme, such as CAT, POX and SOD. Foliar application of tricyclazole significantly alleviated the growth-inhibiting effects of waterlogging stress, evident from increased antioxidant enzyme activity and decreased MDA content and

Table 1. The mean squares of ANOVA for the catalase (CAT), peroxidase (POX) and superoxide dismutase (SOD) activities and malondialdehyde (MDA) content in the leaves of canola plants.

S.O.V.	df	CAT activity (U mg^{-1} protein)	POX activity (U mg^{-1} protein)	SOD activity (% of photoinhibition)	MDA content (nmol g^{-1} FW)
R	2	0.015 ns	0.299 ns	22.096 ns	0.217 ns
T	13	4.906 **	7.843 **	190.567 **	5.924 **
E	26	0.188	0.122	9.772	0.076
CV%		4.799	6.146	5.438	2.808

Note. ** – $p < 0.01$, ns – $p > 0.05$. R – Replication (Block), T – Treatment, E – Error, CV – Coefficient of Variation

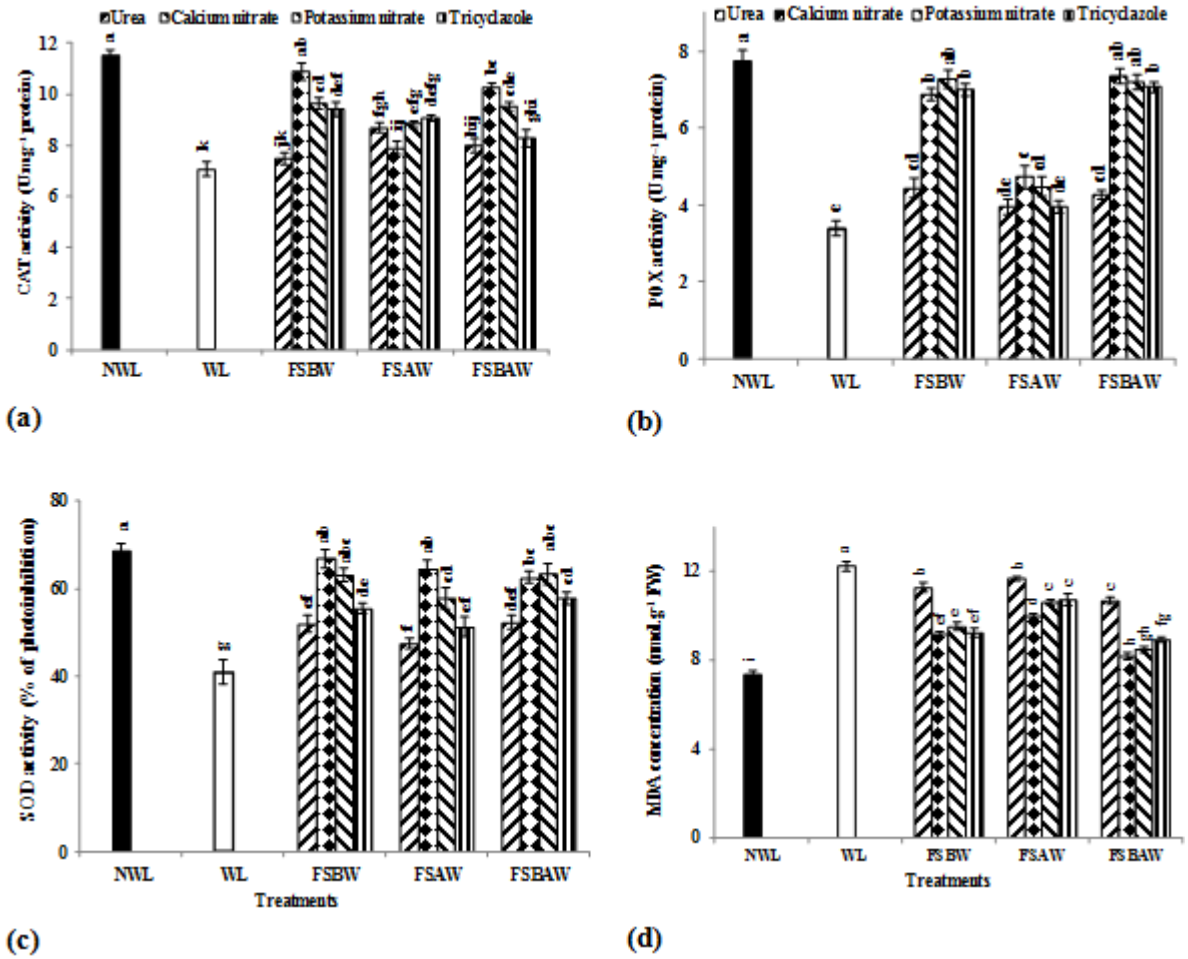

Fig 1. The effect of foliar application of urea, calcium nitrate, potassium nitrate and tricyclazole on canola plants. (a) Catalase (CAT) activity. (b) Peroxidase (POX) activity. (c) Superoxide dismutase (SOD) activity. (d) Malondialdehyde (MDA) content in the leaves. (NWL = non-waterlogged control, WL = waterlogged control, FSBW= foliar spray before waterlogging, FSAW = foliar spray after waterlogging and FSBAW = foliar spray before and after waterlogging). The values are the mean ± standard error (n = 3), and the values followed by the same letter are not statistically different ($P < 0.05$).

ethylene production in leaves. There are different mechanisms for stress-resistance induced by triazoles, they are as follows: effect on isoprenoid pathway; altering levels of plant hormones by inhibition of gibberellin synthesis, reducing ethylene evolution and increasing cytokinin levels (Kamoutsis and Chronopoulou-Sereli 1999); increased chlorophyll content; enlarged chloroplasts; thickening leaf tissue (Watson and Himelick 2004); enhancing both non-enzymatic and enzymatic antioxidant potentials (Kishorekumar et al., 2008). Research has shown that uniconazole could significantly increase antioxidant activity (Leul and Zhou 1999; Zhang et al., 2007). Tests done in this study clarified the effect of tricyclazole on increasing antioxidant activity; reducing MDA content and ethylene production in leaves and eventually boosting the growth of waterlogged canola.

Materials and Methods

Experimental design and treatments

This experiment was accomplished in a greenhouse during the winter of 2011 at Sari Agricultural Sciences and Natural Resources University (53º 13′ E and 36º 42′ N), Sari, Mazandaran, Iran, in a randomized complete block design with 14 treatments and three replications. Canola seeds (cv. Hayola 401) were planted in plastic pots (37 cm diameter and 45cm depth) containing 35 kg of clay loam soil. The treatments included waterlogged (WL) control, non-waterlogged (NWL) control and three different times of foliar applications [i.e. foliar spray before waterlogging (FSBW), foliar spray after waterlogging (FSAW) and foliar spray before and after waterlogging (FSBAW)] of four various

Table 2. The mean squares of ANOVA for the ethylene production in the leaves, root dry weight, shoot dry weight, root length and shoot length of canola plants.

S.O.V.	df	Ethylene production (nl g FW/ h)	Root dry weight (g)	Shoot dry weight (g)	Root length (cm)	Shoot length (cm)
R	2	0.003 ns	0.002 ns	0.007 ns	0.167 ns	0.104 ns
T	13	0.085 **	0.027 **	0.133 **	11.470 **	22.045 **
E	26	0.001	0.001	0.006	0.640	0.630
CV%		5.007	6.542	7.139	5.073	4.887

Note. ** – $p < 0.01$, ns – $p > 0.05$. R – Replication (Block), T – Treatment, E – Error, CV – Coefficient of Variation

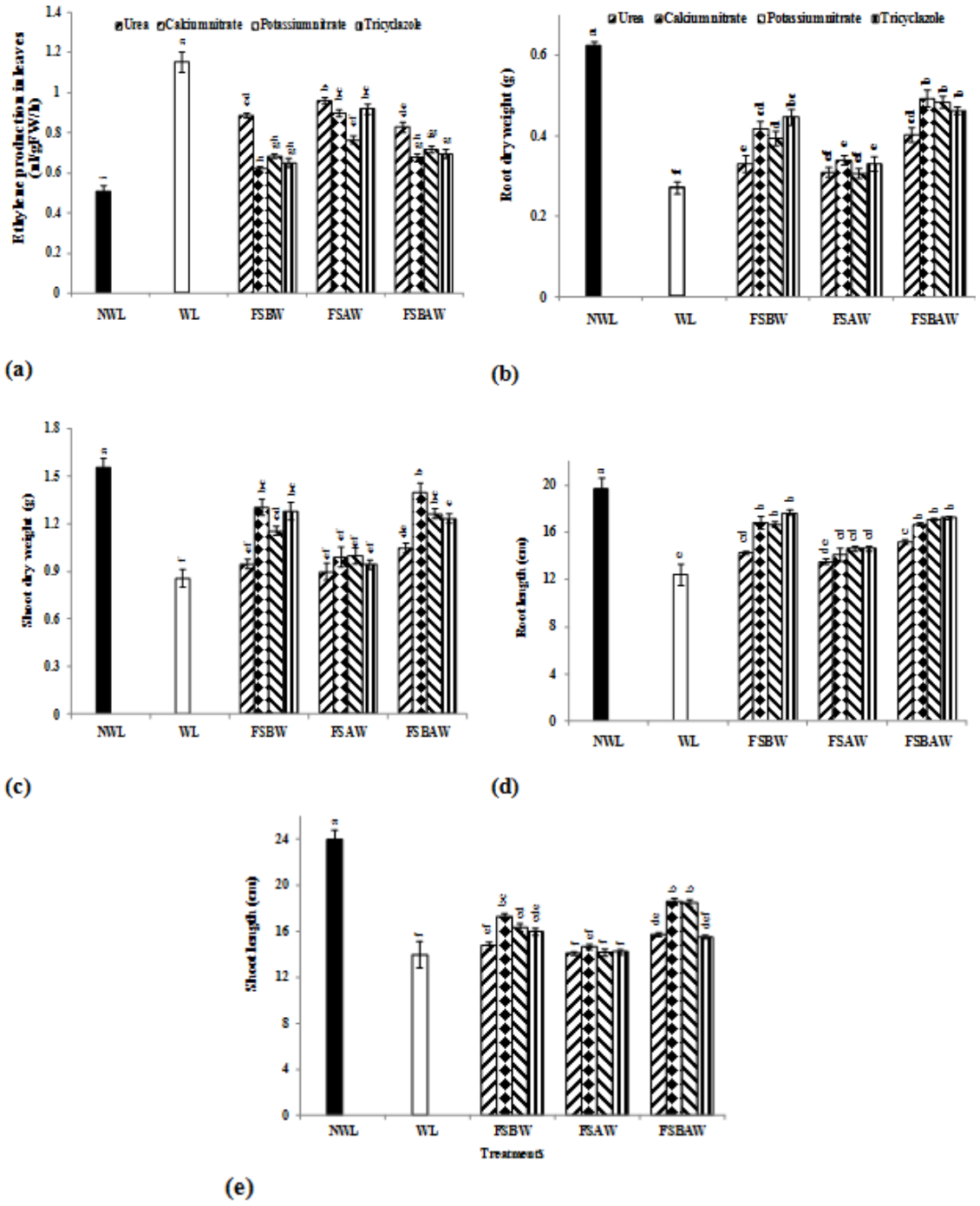

Fig 2. The effect of foliar application of urea, calcium nitrate, potassium nitrate and tricyclazole on canola plants. (a) Ethylene production in the leaves. (b) Root dry weight. (c) Shoot dry weight. (d) Root length. (e) Shoot length. (NWL = non-waterlogged control, WL = waterlogged control, FSBW = foliar spray before waterlogging, FSAW = foliar spray after waterlogging and FSBAW = foliar spray before and after waterlogging). The values are the mean ± standard error (n = 3), and the values followed by the same letter are not statistically different ($P < 0.05$).

compounds (including urea, calcium nitrate, potassium nitrate and tricyclazole). In total, there were 42 pots in three replications, which each replication had 14 pots. Urea, calcium nitrate [$Ca(NO_3)_2$] and potassium nitrate (KNO_3) were used at concentrations of 1500, 4100 and 5000 mg L^{-1}, respectively. As nitrogen contents were not the same in three nitrogen compounds, the foliar applications were adjusted in a way that the nitrogen concentrations were the same in all applications. Meanwhile, tricyclazole was applied at the concentration of 50 mg L^{-1}. Waterlogging stress was applied by putting each pot (except for the NWL control) into a larger plastic bucket (40 cm diameter and 48 cm depth) and filling it with water up to 2 cm above the soil surface at the 5-leaf growth stage for two-week duration.

Enzyme extraction and assays

Fresh leaves (after washing) were frozen in liquid N_2 and stored at -80°C pending biochemical analysis. Frozen leaves (0.2 g) were homogenized in a mortar and pestle with 3 ml ice-cold extraction buffer (25 mM sodium phosphate buffer, pH 7.8). The homogenate was centrifuged at 18,000 x g for 30 min at 4°C, and the supernatant was passed through filter paper to determine enzyme activity and protein content. All procedures were performed at 4°C. Catalase (CAT) activity was estimated by the method cited by Cakmak and Horst (1991). Decrease in absorbance was recorded at 240 nm for 1 min using a Biowave II spectrophotometer (Biochrom Ltd., Cambridge, UK). The catalase activity of the extract was expressed as the $\Delta A\ mg^{-1}$ protein min^{-1}. Peroxidase enzyme (POX) activity was determined by the oxidation of guaiacol in the presence of H_2O_2 (Ghanati et al., 2002). Increase in absorbance at 470 nm was recorded using a spectrophotometer for 1 min, and the POX activity of the extract was expressed as the $\Delta A\ mg^{-1}$ protein min^{-1}. Protein content of the crude extract was determined using bovine serum albumin (BSA) as a standard, according to the method of Bradford (1976). Bradford solution (1 ml) was added to 100 µl crude extract and the absorbance was measured at 595 nm to estimate the total protein content. Protein concentration was calculated using a BSA standard curve. Superoxide dismutase (SOD) activity was determined by measuring the ability of an enzyme extract to inhibit photochemical reduction of nitro-bluetetrazolium (NBT), according to the method cited by Giannopolitis and Ries (1977). Glass test tubes containing the reaction mixture were illuminated with a fluorescent lamp (120 W) and identical tubes that were not illuminated served as blanks. After illumination for 15 min, amounts of absorbance were measured at 560 nm. One unit of SOD activity was defined as the amount of an enzyme that caused a 50% inhibition of the photochemical reduction of NBT.

Determination of lipid peroxidation and ethylene production

Evaluation of malondialdehyde (MDA) was determined according to the method described by De Vos et al. (1991). Briefly, the samples were homogenized in 10% trichloroacetic acid (w/v), and aliquots of the filtrates were heated (95° C for 30 min) in 0.25% thiobarbituric acid. The amount of MDA was measured spectrophotometrically based on absorbance at 532 nm, followed by a correction for non-specific absorbance at 600 nm. The concentration of MDA was determined using the extinction coefficient of MDA (e = 155 µM cm^{-1}). For ethylene measurement**,** leaf samples were placed in a 60 ml culture tube, which was sealed with a rubber stopper. Tubes were incubated for 2 h at 25°C, and a 1.0 ml sample of the headspace gas was removed using a hypodermic syringe and analyzed for ethylene using a gas chromatograph (Model GM-816, GOW MAC Instrument CO., Bridgewater, New Jersey, USA) equipped with an Al_2O_3 column and hydrogen flame ionization detector (Dong et al., 1983).

Shoot and root sampling

Plant samples were collected two weeks after the end of the waterlogging stress period. In order to avoid damaging the roots when they were pulled out, all pots were temporarily waterlogged for 1 h. After careful uprooting, plant samples were divided into their various parts; the shoots (aboveground parts) and roots (belowground parts). Samples were washed three times with deionized water and measurements were taken for plant and root lengths (cm). To determine the dry weight, the shoots and roots were oven-dried separately at 70°C for 72 h.

Data Analysis

All data were subjected to analysis of variance and means were separated by Duncan's multiple range tests using SAS software.

Conclusions

This is the first report done to compare the useful effect of the foliar application of different nitrogen compounds and tricyclazole on plant tolerance to waterlogged condition. In this investigation, applications of urea, calcium nitrate, potassium nitrate and tricyclazole significantly alleviated the growth-inhibiting effects of waterlogging stress. The foliar spray of calcium nitrate, potassium nitrate and tricyclazole before waterlogging stress was found to be the most superior. It appears that due to foliar spray before the stress, the plants had sufficient time to use and metabolize the absorbed compounds. Thus, the damage of waterlogging stress on plants was limited. Although foliar application before waterlogging or before and after waterlogging produced similar effects by alleviating damage from waterlogged conditions, it may be concluded that foliar spray before waterlogging was more advisable (due to the cost efficiency) to enhance tolerance to waterlogging stress in canola.

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