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A comparison between foliar application and seed inoculation of biofertilizers on canola (*Brassica napus* L.) grown under waterlogged conditions

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Abstract

Waterlogging restricts canola growth via different physiological changes. The objective of this work was to compare the effects of the foliar and seed application of two biofertilizers on some physiological and morphological responses in canola plants (*Brassica napus* L. cv. Hayola 401) under waterlogging stress conditions. Plants at 5-leaf stage were exposed to flooding conditions for two weeks. Two biofertilizers; AAP (*Azotobacter chroococcum*, *Azospirillum spp.* and *Pseudomonas spp.*) and APB (*Azospirillum spp.*, *Pseudomonas fluorescens* and *Basillus subtilis*) were applied by seed inoculation or foliar spray at different times i.e. before waterlogging, after waterlogging and, before and after waterlogging. The results showed that the flooding stress significantly decreased the dry weight and length of the shoots and roots. The activity of superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX) were reduced, whereas lipid peroxidation and ethylene production in the leaves were increased under waterlogging stress. The adverse effects of the flooding stress were significantly alleviated by the seed inoculation and foliar application once (before the stress) of both biofertilizers compared to the waterlogged control. However, among two methods, inoculating the seeds with the biofertilizers is cost efficient and advisable to alleviate waterlogging damage in canola.

Keywords: Antioxidant activity; Biofertilizer; Canola; Oxidative stress; Waterlogging.
Abbreviations: ACC-1-amino cyclopropane-1-carboxylic acid; AcdS-ACC deaminase; ACO-ACC oxidase; ACS-ACC synthase; 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; PGPR-plant growth promoting rhizobacteria; NBT-nitrobluetetrazolium; POX-peroxidase; ROS-reactive oxygen species; SOD-superoxide dismutase.

Introduction

Canola (*Brassica napus* L.) is a world's major oilseed crops and important source of edible oil. It is expanding rapidly as a rotation crop following rice. Among the abiotic stresses, waterlogging is major constraint for production and productivity of most of the crops (Zhou and Lin 1995). Waterlogging or an excess of water availability is a global crop production constraint that causes significant yield reductions in canola (Zhou, 1994). Such yield reductions may occur after 3 to 30 days of flooding stress, depending on the climate and the developmental stage of the plants (Gutierrez Boem et al., 1996). Zhou and Lin (1995) reported that the physiological reactions to waterlogging at the seedling and floral bud appearance stages of canola were associated with decreases in the leaf chlorophyll content, superoxide dismutase (SOD) and catalase (CAT) activities, root oxidizability, plant height, accumulation of leaf malondialdehyde (MDA), a greater ethylene production, and a reduction in leaf photosynthetic rate. A lack of O_2 due to waterlogging may limit the crop growth because of alterations in metabolism (Drew, 1992) and the nutrient uptake of plants, leading to the generation of reactive oxygen species (ROS). These toxic oxygen species react with numerous cell components and cause oxidative stress (Scandalios, 1993). Plants may respond to waterlogging by altering their hormone balance and the growth of stems and roots (Grichko and Glick, 2001b). Indeed, the ethylene production in the shoot is responsible for the abnormal growth of plants under waterlogged conditions (Saleem et al., 2007). Seed inoculation and the foliar spray of biofertilizers, products containing plant growth-promoting rhizobacteria (PGPR), have been used to reduce or eradicate the negative effects of ethylene under stress conditions (Wu et al., 2005; Saleem et al., 2007). Basha et al. (2006) found that the application of PGPR as a foliar spray provided a superior efficiency in the management of fungal diseases on chickpea, and Esitken et al. (2006) reported a significant effect of a PGPR foliar spray on the yield increases of sweet cherry. Vijayan et al. (2007) founded the better performance of foliar application of *Azotobacter chroococcum* in alleviating the growth-inhibiting effects of salinity in mulberry plants, and Grichko and Glick (2001a) reported that tomato plants inoculated with PGPR showed a substantial tolerance to waterlogging stress. However, evidences are limited for comparison of the effects of foliar and seed applications of PGPR on canola under waterlogged stress conditions. Therefore, the objective of this work was to compare the effects of foliar application and seed inoculation of two

biofertilizers on some physiological and morphological responses of canola plants to waterlogging stress.

Results

Antioxidant activity

The waterlogging caused a significant decrease in the catalase (CAT) activity of the canola leaves compared with the non-waterlogged (NWL) control (Fig 1a). The seed inoculation (SI), foliar spray before waterlogging (FSBW) and foliar spray before and after waterlogging (FSBAW) treatments of both biofertilizers significantly increased the CAT activity compared to the waterlogged (WL) control, and there was no significant difference between SI, FSBW and FSBAW. In contrast, the application of the biofertilizers after waterlogging stress had no significant effect on the CAT activity compared to the WL control. The peroxidase (POX) activity was significantly decreased by the waterlogging stress when compared with the NWL (Fig 1b). The SI, FSBW and FSBAW treatments of both biofertilizers significantly increased the POX activity compared to the WL control, so that there was no significant difference among them. Similar to CAT, the foliar spray of the biofertilizers after waterlogging stress had no significant effect on the POX activity in comparison with WL control. The waterlogging stress significantly decreased the superoxide dismutase (SOD) activity in the canola leaves, compared with the NWL control (Fig 1c), and the foliar spray of both biofertilizers before waterlogging or before and after the stress resulted in a significantly higher SOD activity. The foliar spray after waterlogging caused the least significant increase in the SOD activity compared with the WL control.

Lipid peroxidation

The concentration of malondialdehyde (MDA), as an indicator of lipid peroxidation, was significantly increased in the canola leaves due to waterlogging stress (Fig 1d). The SI, FSBW and FSBAW treatments of biofertilizers significantly decreased the MDA concentration compared to the WL control. However, the foliar spray of biofertilizers after the waterlogging stress did not significantly change the MDA concentration when compared with the WL control. Furthermore, no difference between the biofertilizer treatments was observed.

Ethylene production in the leaves

The waterlogging caused a significant increase of ethylene production in the leaves, compared with the NWL control (Fig 2). The SI, FSBW and FSBAW treatments of biofertilizers significantly decreased the ethylene production in the leaves compared to the WL control. The foliar spray after waterlogging (FSAW) of the AAP biofertilizer (*Azotobacter chroococcum, Azospirillum spp.* and *Pseudomonas spp.*) significantly decreased the ethylene level compared to the WL control. The effect for the FSAW of the APB biofertilizer (*Azospirillum spp.*, *Pseudomonas fluorescens* and *Basillus subtilis*) was not significantly different from the WL control.

Root and shoot dry weights and lengths

The root dry weight significantly decreased after the waterlogging stress (Fig 3a). The SI, FSBW and FSBAW treatments of biofertilizers significantly increased the root dry weight, compared with the WL control, and no significant differences among them were observed. The foliar application of biofertilizers after waterlogging stress did not affect the root dry weight when compared with the WL control. There was no difference between the biofertilizers in the all applications. The root length was also significantly decreased due to the waterlogging stress (Fig 3b). The seed inoculation of biofertilizers resulted in a significantly longer root length, and the effect of FSBW and FSBAW on the increase of root length was less pronounced than the seed inoculation. There was no significant difference between biofertilizers. The waterlogging stress significantly decreased the shoot dry weight (Fig 3c), whereas the seed inoculation of the biofertilizers caused a significant increase in the shoot dry weight in comparison with the WL control. FSBW and FSBAW had lower effects on the increase in the shoot dry weight. The effect of FSAW was not significant, and there was no difference between the biofertilizers in any of the applications. The shoot length was also significantly decreased by the waterlogging stress (Fig 3d). The effects of the SI, FSBW and FSBAW treatments of biofertilizers were significant on the increase in the shoot length. Similar to the above results, the effect of FSAW was not significant when compared with the NWL control, and there was no significant difference between the biofertilizers.

Discussion

Waterlogging leads to oxidative stress in plants through an increase in the reactive oxygen species. It is known that ROS trigger a series of deleterious processes, such as lipid peroxidation, degradation of proteins, and DNA damage in the cell (Scandalios, 1993). Thus, the physiological and biochemical processes of plants are altered by waterlogging stress (Jackson and Colmer, 2005). Because of higher antioxidant activities, less ROS accumulates in tolerant plants and, as a result, the oxidative damage is reduced. Therefore, waterlogging stress resistance may depend, at least in part, on the enhancement of the activity of antioxidant enzymes, such as CAT, POX and SOD, which are a part of the antioxidative defense system. All of these enzymes play important roles in scavenging harmful ROS (Hideg, 1997). The malondialdehyde content is often used as an indicator of the lipid peroxidation in plant tissues that results from oxidative stress induced by various abiotic stresses (Tang et al., 2010). In this study, changes in activity of antioxidant enzymes and the MDA content indicated that oxidative stress is an important component of waterlogging stress in canola, so that reduction in the shoot and root growth could be a consequence of the oxidative stress induced by the waterlogging conditions. Waterlogging stress causes plants to disrupt hormonal balance (Grichko and Glick, 2001b). In the current study, the significant increase of ethylene production in the leaves due to waterlogging stress may be responsible, at least partly, for reduction of growth and inducing leaf senescence. Decreasing the root and shoot dry weight and enhancing MDA content in the leaves are evidences for diminution of growth and induced leaf senescence, respectively. The overproduction of ethylene in response to abiotic and biotic stresses leads to inhibition of root growth and, consequently, inhibition of the growth of the entire plant under stress conditions (Bleecker and Kende, 2000; Saleem et al., 2007). Ethylene is derived from the amino acid, methionine, which is converted to S-adenosylmethionine (SAM) by SAM synthetase. Accordingly, SAM is usually considered as the earliest precursor of ethylene and is first converted into 1-amino cyclopropane-1-carboxylic acid

(ACC) by the activity of ACC synthase (ACS, EC: 4.4.1.14). The final step of ethylene biosynthesis, the conversion of ACC to ethylene, is catalyzed by the ACC oxidase enzyme (ACO or ethylene-forming enzyme; EC: 1.14.17.4) (Adams and Yang, 1979). The positive effect of biofertilizers containing PGPR in this experiment can have different justifications. It may be resulted from the encouraging effect of biofertilizers on plant growth. PGPR are notable for their ability to trigger increased biomass production and crop yield (Vessey, 2003). The inoculation of plant roots with efficient PGPR strains usually enhances the lateral root proliferation and root hair elongation (German et al., 2000). The ability of many rhizobacteria to produce plant hormones or hormone-like substances has often been evoked to explain how PGPR can promote plant growth (Bloemberg and Lugtenberg, 2001); it also has been proposed that the PGPR could affect the hormones level in the host plant. For instance, PGPR can lower ethylene levels, thus partially relieving the negative regulation exerted on plant growth by this gaseous hormone (Ma et al., 2002). In this study, the significant decrease of ethylene production in the leaves of waterlogged-canola due to application of biofertilizers may be justified by the ability of PGPR to lower ethylene levels in plant. Two mechanisms, including rhizobitoxine excretion and ACC deaminase activity, for the role of PGPR in lowering ethylene levels have been proposed. Rhizobitoxine is a toxin that inhibits ACS activity (Yuhashi et al., 2000), and the synthesis of rhizobitoxine has been identified in only in a few bacteria belonging to the Bradyrhizobium (Yasuta et al., 2001) and the Pseudomonas genera (Mitchell and Coddington, 1991). ACC deaminase (AcdS, EC: 3.5.99.7) catalyzes the degradation of ACC into α-ketobutyrate and ammonia. Since its discovery, AcdS activity and/or the gene has been found in many microorganisms, including a large range of bacteria, especially such soil-living bacteria associated with plants roots as Achromobacter, Agrobacterium, Azospirillum, Burkholderia, Enterobacter, Pseudomonas, Rastolnia and Rhizobium (Hontzeas et al., 2005). It has been postulated that much of the ACC produced by the ACS activity in plant roots may be exuded into the rhizosphere. The ACC could then be taken up by the rhizobacteria and hydrolyzed by AcdS. The PGPR that express ACC deaminase regulate and lower the levels of ethylene. These ACC deaminase-producing PGPR boost plant growth, particularly under conditions of stress, by the regulation of accelerated ethylene production in response to a multitude of abiotic and biotic stresses, such as salinity, drought, waterlogging, temperature, pathogenicity and contaminants (Glick et al., 1998).

Materials and methods

Experimental design and treatments

The experiment was conducted during the winter of 2011 in a greenhouse at Sari Agricultural Sciences and Natural Resources University (53º 13′ E and 36º 42′ N), Sari, Mazandaran Province, Iran. The experiment was arranged in a completely randomized design with 10 treatments, and each treatment was replicated three times. The seeds of canola (*Brassica napus* L.) cv. Hayola 401 were sown in plastic pots (37 cm diameter and 45 cm depth) containing approximately 35 kg of clay loam soil (36 % clay, 41 % silt and 23 % sand). 10 seeds were sown in each pot, and after full germination, the number of plants was reduced to four seedlings per pot. The plants were irrigated at the field capacity level. The treatments included waterlogged (WL) and non-waterlogged (NWL) controls. Four levels of biofertilizer applications viz. seed inoculation (SI); foliar spray before waterlogging (FSBW) at the 3-leaf growth stage; foliar spray after waterlogging (FSAW) and foliar spray before and after waterlogging (FSBAW). Two biofertilizers used in study were AAP (*Azotobacter chroococcum*, *Azospirillum brasilense*, *A. lipoferum*, *Pseudomonas fluorescens* and *P. putida*) and APB (*Azospirillum brasilense*, *A. lipoferum*, *Pseudomonas fluorescens* and *Basillus subtilis*) produced by Biotechnology Institute, Tehran, Iran. The bacterial concentration of each biofertilizer was 10^8 CFU ml^{-1}.

All pots (except for the NWL control) were uniformly subjected to waterlogging stress at the 5-leaf growth stage for two weeks. To apply the waterlogging treatments, each pot was placed into a plastic bucket (40 cm diameter and 48 cm depth). The waterlogging treatments were then applied by filling the outer container with water up to 2 cm above the soil surface.

Enzyme extraction and assays

After 2 weeks of the waterlogging treatment, fresh leaves (after washing) were frozen in liquid N_2 and stored at -80°C until the 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 (crude extract) was passed through filter paper and used for the determination of the enzyme activity and protein content. All of the procedures were performed at 4°C. The catalase (CAT) activity was estimated by the method of Cakmak and Horst (1991). The decrease in the 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 ΔA mg^{-1} protein min^{-1}. The peroxidase enzyme (POX) activity was determined by the oxidation of guaiacol in the presence of H_2O_2 (Ghanati et al., 2002). The increase in the absorbance at 470 nm was recorded using a spectrophotometer for 1 min, and the POX activity of the extract was expressed as the ΔA mg^{-1} protein min^{-1}. The 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. The protein concentration was calculated using a BSA standard curve. The superoxide dismutase (SOD) activity was determined by measuring the ability of the enzyme extract to inhibit the photochemical reduction of nitrobluetetrazolium (NBT), according to the method of Giannopolitis and Ries (1977). Glass test tubes that contained 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, the absorbance was measured at 560 nm. One unit of SOD activity was defined as the amount of enzyme that caused a 50% inhibition of the photochemical reduction of NBT.

Determination of lipid peroxidation

The level of membrane damage was determined by measuring the amount of malondialdehyde (MDA), which is the end product of lipid peroxidation (De Vos et al., 1991). In brief, 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 the

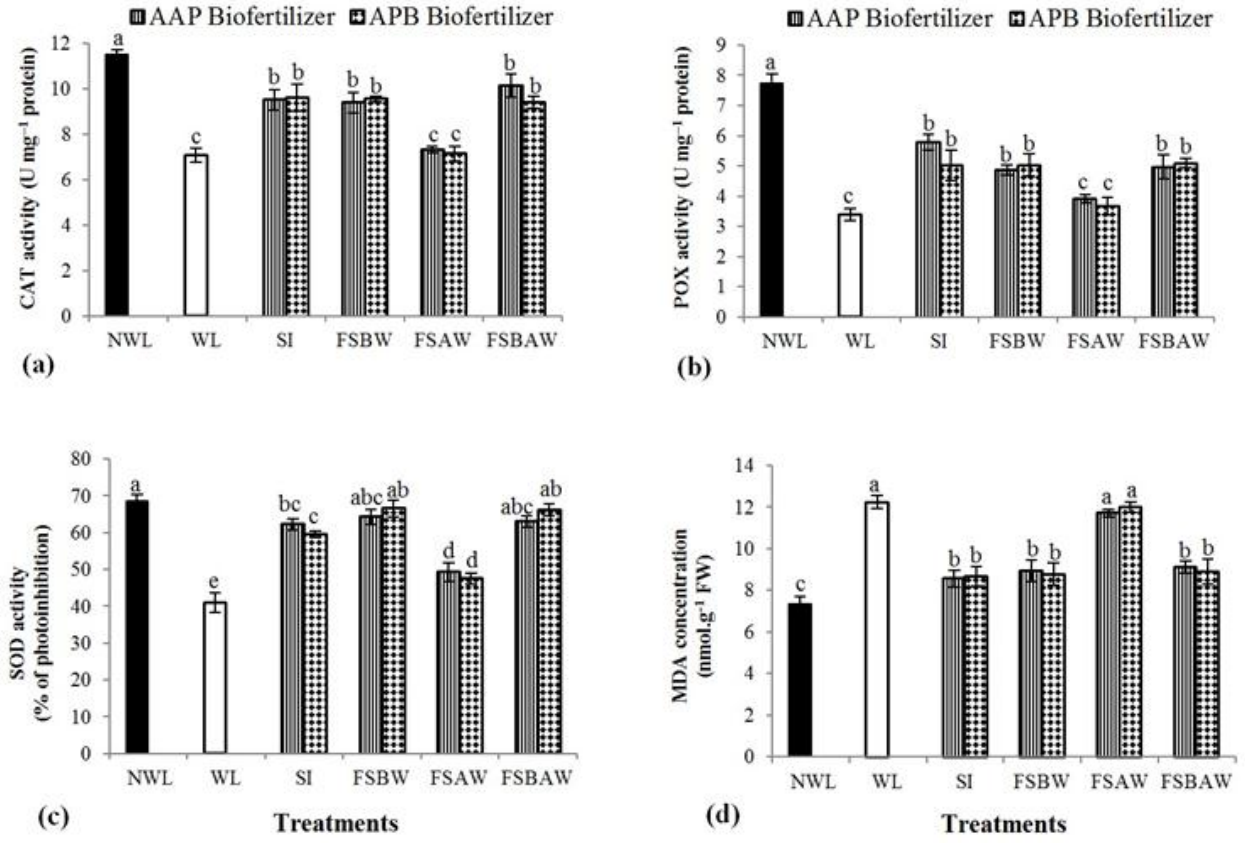

Fig 1. The effect of biofertilizers and application methods on the catalase (CAT) activity (a), peroxidase (POX) activity (b), superoxide dismutase (SOD) activity (c) and malondialdehyde (MDA) content (d) in the leaves (NWL = non-waterlogged control, WL = waterlogged control, SI = seed inoculation, FSBW = foliar spray before waterlogging, FSAW = foliar spray after waterlogging and FSBAW = foliar spray before and after waterlogging). The values are the mean ± SE (n = 3), and the values followed by the same letter are not statistically different ($P < 0.05$).

absorbance at 532 nm, followed by a correction for the non-specific absorbance at 600 nm. The concentration of MDA was determined using the extinction coefficient of MDA (e = 155 µM cm^{-1}).

Ethylene production measurement

The ethylene production was measured using gas chromatography (Dong et al., 1983). The leaf samples were placed in a 60 ml culture tube, which was sealed with a rubber stopper. The 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.

Shoot and root sampling

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

Data analysis

All of the data were subjected to an analysis of variance (ANOVA), and the means were separated by Duncan's multiple range tests using SAS software.

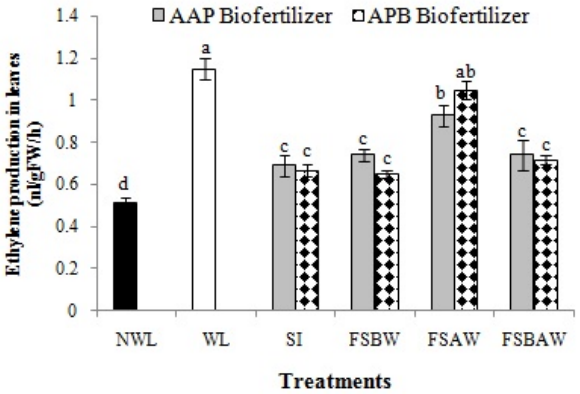

Fig 2. The effect of biofertilizers and application methods on the ethylene production in the leaves (NWL = non-waterlogged control, WL= waterlogged control, SI= seed inoculation, FSBW= foliar spray before waterlogging, FSAW = foliar spray after waterlogging and FSBAW= foliar spray before and after waterlogging). The values are the mean ± SE

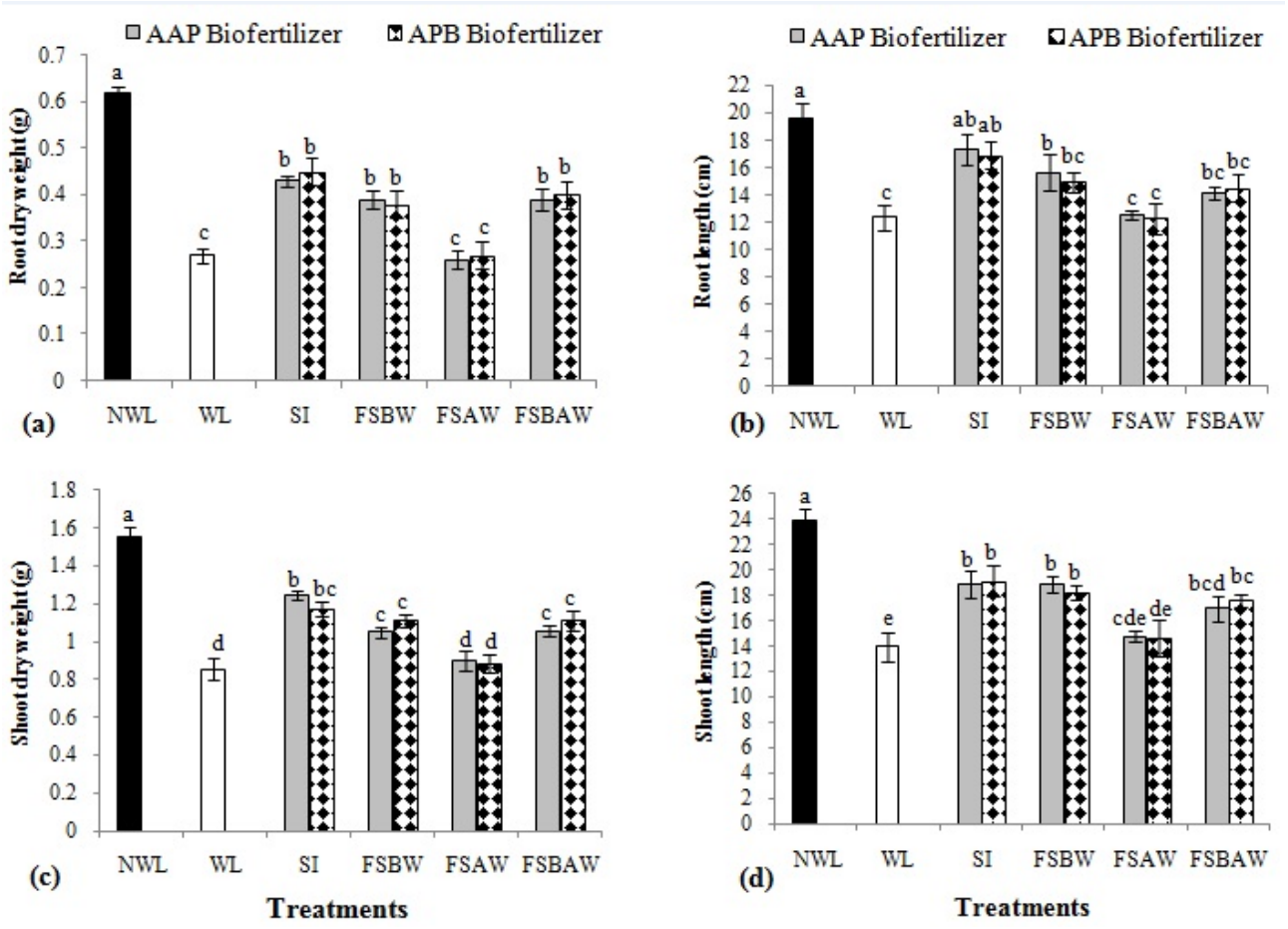

Fig 3. The effect of biofertilizers and application methods on the root dry weight (a), root length (b), shoot dry weight (c) and shoot length (d) of canola plants (NWL = non-waterlogged control, WL= waterlogged control, SI= seed inoculation, FSBW= foliar spray before waterlogging, FSAW = foliar spray after waterlogging and FSBAW= foliar spray before and after waterlogging). The values are the mean ± SE (n = 3), and the values followed by the same letter are not statistically different ($P < 0.05$).

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Conclusions

This is the first report regarding the useful effect of the foliar application of biofertilizers on waterlogging tolerance in canola. In the present investigation, the application of biofertilizers by two methods significantly alleviated the growth-inhibiting effects of waterlogging stress, as evinced by the increased the antioxidant enzyme activities and decreased MDA content and ethylene production in the leaves.

The performance of biofertilizers could be explained by the fixation of sufficient atmospheric nitrogen by the bacteria, the production of plant growth promoters, such as auxins, gibberellins and cytokinins (Vasantharajan and Bhatt, 1968; Saxena and Tilak, 1994), and reduction of ethylene production in plants. Among the different methods of application, seed inoculation and the foliar spray of biofertilizers before waterlogging stress were found to be superior. It appears that by using the foliar spray before the stress, the plants had sufficient time to utilize the beneficial products generated from the activity of the PGPR. Thus, the damage of flooding stress on the plants was reduced. The biofertilizers applied by foliar spraying resulted in relatively equal effect in comparison with the inoculation of the seeds. Although both application methods produced similar effects by alleviating the waterlogging damage, it may be concluded that the seed inoculation of the biofertilizer is the advisable method to enhance tolerance to flooding stress in canola because it is easier to use and more cost efficient than the foliar application. These results provide new evidence in the elucidation of the mechanism that underlies the ability of biofertilizers to help plants tolerate waterlogging damage, hence future experiments based on these criteria may aid in the development of biofertilizer applications to mitigate the deleterious effects of waterlogging.

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