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Alleviation of allelochemical stress-induced growth inhibition and oxidative damage in lettuce under closed hydroponics through electro-degradation

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Abstract: Successive lettuce cultivation in closed hydroponics using the same nutrient solution causes the excess production and accumulation of allelochemicals. The accumulated allelochemicals induce oxidative damage and lipid peroxidation in plants leading to growth inhibition. In this study, we investigated the allelochemicals that induced oxidative damage and lipid peroxidation in lettuce grown in a once used non-renewed nutrient solution (1NR) and a twice used non-renewed nutrient solution (2NR) obtained from the successive cultivation and the alleviation of these damages through electro-degradation (ED). The 1NR solution was used for six weeks for a one-time lettuce cultivation while the 2NR solution was used for twelve weeks for a two-times lettuce cultivation. The results showed that the allelochemical stress caused growth inhibition in the lettuce in both the 1NR and 2NR solutions. It was observed that there was a higher generation of H_2O_2 and $O_2^{\cdot -}$ as well as a lower activity of the antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (POD), and ascorbate peroxidase (APX) in the roots of the plants grown in both the 1NR and 2NR solutions compared to plants grown in the new nutrient solution. The higher level of lipid peroxidation due to the higher MDA (malondialdehyde) content and higher soluble protein content were also observed in the roots of those plants. It was evident that lettuce root damage occurred due to accumulation of the allelochemicals in the 1NR and 2NR solutions. These damaged roots could not function normally nor uptake water and minerals from the culture solution. As a result, retarded lettuce growth was observed in the 1NR and 2NR solutions. The oxidative damage, soluble protein content, lipid peroxidation and ultimately growth retardation were more pronounced in the plants grown in the 2NR solution compared to the plants grown in the 1NR solution. The application of ED to the 1NR and 2NR solutions maintained the plant growth through less oxidative damage, soluble protein production and lipid peroxidation as was observed in the plants grown with the new nutrient solution. Therefore, the ED of a non-renewed culture solution would alleviate the allelochemical stress in lettuce under recycled hydroponics.

Keywords: lettuce; closed hydroponics; enzyme reactions; allelochemical stress; oxidative damage

In closed hydroponics, the accumulation of allelochemicals occurs in the culture solution due to root exudations (Inderjit, Weston 2003). When these compounds suppress plant growth, the phe-

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nomenon is considered to be a biotic stress termed "allelochemical stress" (Cruz-Ortega et al. 2002). When it occurs among the individuals of the same species due to their root exudation, it is called autotoxicity. In closed hydroponics, this stress has been documented in a number of crop species due to the accumulated root exudates in the rhizosphere (Tang, Young 1982; Singh et al. 1999; Kitazawa et al. 2005; Asao et al. 2007, 2008; Asaduzzaman et al. 2012) including lettuce (Asao et al. 2004b; Lee et al. 2006). In this phenomenon, the lettuce root secretes several allelochemicals to the culture solution causing damage to the root cells, which, in turn, hampers water and mineral nutrient absorption resulting in growth inhibition and yield loss. In successive closed hydroponic lettuce cultivation using the same nutrient solution, the plant growth was greatly reduced and root injury increased due to presence of several allelochemicals in the reused nutrient solution. Growth retardation and root injury gradually increased with the increased times the nutrient solution was reused (Lee et al. 2006).

The removal or degradation of these phytotoxic substances that have accumulated in the nutrient solution would lead to a normal growth and crop yield. In these regards, the nutrient solution that feeds the plants needs to be periodically replaced. However, this process requires more labour, time, money and generates hydroponic wastewater that is particularly rich in nitrogen and phosphorus; when these nutrients are discharged directly into the environment, they may cause contamination (Bertoldi et al. 2009). As a result, many researchers tried to remove or degrade these accumulated allelochemicals in an alternative means such as the adsorption of the allelochemicals by activated charcoal (AC) (Asao et al. 1998; Lee et al. 2006) and Amberlite XAD-4 (Lee et al. 2006), the degradation of the allelochemicals by electro-degradation (ED) means (Asao et al. 2008; Asaduzzaman et al. 2012; Talukder et al. 2019a,b), the degradation of the allelochemicals by microbial strains (Asao et al. 2004a).

When plants are exposed to allelochemical stress in closed hydroponics, they suffer from the disruption of the normal physiological process before the ensuing yield loss. The allelochemical stress alters the ion uptake and hydraulic conductivity (Blum et al. 1999), alters the mineral uptake (Lyu, Blum 1990; Baziramakenga et al. 1994), disrupts the membrane permeability (Baziramakenga et al. 1995), causes stomatal closure and induces water stress (Barkosky, Einhellig 1993), influences respiration (Penuelas et al. 1996), affects photosynthesis and protein synthesis (Mersie, Singh 1993; Rohn et al. 2002), impairs the hormone balance (Holappa, Blum 1991) and alters enzyme activities (Rohn et al. 2002; Doblinski et al. 2003). Similar to other biotic stresses, in an allelopathic reaction, an essential function of the reactive oxygen species (ROS) has been indicated by some authors (Weir et al. 2004; Gniazdowska, Bogatek 2005; Cruz-Ortega et al. 2007). In allelochemical stress, the shift from the regulatory role of the ROS in the cell signalling to their toxicity is probably related to the changes in the homeostasis of the ROS maintained by the imbalance of the ROS production and ROS scavenging. It induces oxidative damage which is evidenced by a high level of lipid peroxidation (Romero-Romero et al. 2005; Lara-Núñez et al. 2006) and the generation of more ROS in the plants (Cruz-Ortega et al. 2002; Weir et al. 2004; Batish et al. 2006; Singh et al. 2006). An undue amount of soluble protein production under stress conditions was observed by several other researchers (Singh et al. 1987; Ashraf et al. 2004). The induction of oxidative stress by allelochemicals was investigated in a range of plants, e.g., the soybean (Böhm et al. 2006), maize (Mylona et al. 2007) and rice (Chi et al. 2011). Other studies also have shown that allelochemical stress can cause oxidative damage to plants (Bais et al. 2003; Sánchez-Moreiras et al. 2005; Abenavoli 2006).

The most common ROS are hydrogen peroxide, superoxide, the hydroxyl radical, and singlet oxygen that formed as a natural by-product of the normal metabolism of oxygen and is crucial in cell signalling. The overproduction of ROS leads to oxidative stress and can cause damage to the cellular components. To diminish the impact of the oxidative stress, plants have evolved a complex system of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (POD), ascorbate peroxidase (APX) and glutathione reductase (GR) and some other non-enzymatic antioxidants, that accumulate in higher plants under stress conditions (Ozkur et al. 2009). Plants enhance antioxidant production in order to minimise the detrimental effects of the oxidative stress to normalise their metabolic activities. The elevated accumulation of antioxidant enzymes, such as SOD, CAT, GR, APX, and POD, is involved in lowering the oxidative damage that was observed in caper bush seedlings under drought stress (Ozkur et al. 2009). In another study, Yang et al. (2009) found an increase in the activity of CAT, SOD, POD, APX,

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and GR under drought stress conditions. In successive closed hydroponic cultivation due to the overproduction of allelochemicals, oxidative imbalance was created by generating more ROS and altering the antioxidant enzyme activity. Elimination of these accumulated allelochemicals may lead to the possibility of maintaining an oxidative balance.

In this study, we investigated the influence of the allelochemical stress on the antioxidant system of lettuce grown in the reused solutions under successive closed hydroponic cultivation. The activities of the antioxidant enzymes, such as CAT, APX, SOD and POD, were studied. We also examined the levels of H_2O_2, $O_2^{\cdot-}$, the soluble protein and membrane damage as lipid peroxidation. Together with other methods, the ED of the culture solutions was also studied for the alleviation of the allelochemical induced growth inhibition and maintaining a balance between the overproduction of ROS and their scavenging by the enhanced production of CAT, SOD, POD, and APX.

MATERIALS AND METHODS

Plant material

In this study, lettuce (*Lactuca sativa* cv. Southern) was used as the plant material. The seeds were (Takii seed company, Japan) sown in cell trays (48 cm × 24 cm × 4 cm, 72 cells/tray) with a vermiculite substrate [Size: Medium (1~5mm); Origin: Asahi Industrial Co. Ltd., Okayama, Japan] and were kept in a growth chamber at 25/20 °C (day/night), 60% relative humidity, with a fluorescent light with an intensity of 140~160 μmol/m^2/s and a 12-hour photoperiod. After two weeks, the seedlings were transferred to the grow beds of the hydroponic system in plastic containers (68 cm × 53 cm × 23 cm) for the nursery in an environmental control room. The room was maintained at a relative humidity of 60%, a temperature of 20/20 °C (day/night), a CO_2 concentration of 800 ppm, with a fluorescent light with an intensity of 145 μmol/m^2/s and a photoperiod of 12 hours. One hundred seedlings were accommodated in each grow bed and 30 L of 50% standard "Enshi" nutrient solutions were used for each hydroponic system and the solution was renewed weekly. Continuous aeration was maintained in the nursery by a pump (MX 808ST-W, Enomoto, Micro Pump Mfg. Co. Ltd., Japan; Flow rate 25 l/min). The seedlings were kept there for two weeks. Then, the more homogenous seedlings were selected as the planting material.

Nutrient solution

The lettuce seedlings were cultured in a 50% standard "Enshi" nutrient solution (Hori 1966). The pH and EC (electrical conductivity) of the nutrient solutions were 7.15 and 1.4 dS/m, respectively, whereas the values of the tap water used to prepare this nutrient solution were 8.18 and 0.22 dS/m, respectively. The used solutions collected from the successive closed hydroponic lettuce were initially also the 50% standard "Enshi" nutrient solution.

ED of allelochemicals in the used nutrient solution

The collected used nutrient solutions were filtrated through Whatman No. 2 filter paper. The 10 L filtered solution was electro-degraded with an electro-degradation machine. An alternating current type electro-degradation machine (Yonago Shinko Co., Ltd., Tottori, Japan) was used for the ED of the autotoxic chemicals in the used solution. In this machine, the electrode had a central core made of titanium with a surface area of 53.1 cm^2 (anode/cathode) which is enclosed within a cylindrical tube also made of titanium with a surface area of 95.5 cm^2 (cathode/anode) (Talukder et al. 2019a). The nutrient solution could pass through the electrode where the ED took place. The electrodes were coupled with a digital alternating current power supplier (AD-8735D, AND, Japan). During the ED, the following was maintained: 500 Hz, 50% duty ratio, 1.8A and 24V. This process was maintained for 24 hours.

Adsorption of allelochemicals from used nutrient solution by amberlite XAD-4

The amberlite XAD-4 (20–60 mesh) collected from Sigma Co. was used as a good adsorbent of the allelochemicals from the used nutrient solutions. At first, the used nutrient solutions were filtrated through Whatman No. 2 filter paper. Then, the 10 L filtered solution was passed through a glass column (length 15 cm × diameter 5 cm) filled with 100 g of amberlite XAD-4 at a running rate of 7 mL/min.

Adsorption of allelochemicals from used nutrient solution by activated charcoal (AC)

100 g of AC (Sigma Co.) was mixed with the 10 L filtered used nutrient solution and the solution was aerated by a pump (MX 808ST-W, Enomoto, Micro Pump Mfg. Co. Ltd., Japan) for 24 hours and then, the solution was again filtered through Whatman No. 2 filter paper to remove the AC.

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Mineral adjustment in the used nutrient solutions

Before starting the experiment, the major nutrients' (NO_3^-, PO_4^{3-}, K^+, Ca^{2+}, Mg^{2+} and Fe^{3+}) concentration in the used nutrient solution and the treated (ED, XAD-4 and AC) solutions were adjusted as close as possible to the concentration of the new 50% 'Enshi' solution based on the chemical analyses. A small amount of the nutrient solution (25 mL) was collected in plastic bottles for the analyses of the major nutrients. The nutrient solution was filtered with qualitative filter paper (Advantec Grade No. 131; 125 mm). The major mineral nutrients such as K^+, Ca^{2+}, Mg^{2+} and Fe^{3+} were measured with an atomic absorption spectrophotometer (Z-2000, Hitachi High-Technologies Corporation, Kyoto, Japan), NO_3^- was measured with a compact meter TWIN NO_3^- (B-343, Horiba, Ltd., Japan) and PO_4^{3-} was measure using a spectrophotometer at 720 nm (U-2900, Hitachi High Technology, Tokyo, Japan).

Lettuce bioassay using used nutrient solution

Bioassay Using Once Used Nutrient Solution (Bioassay I). The selected seedlings were planted in the plastic containers (30 cm × 20 cm × 10 cm). The containers were filled with 3 L of the nutrient solution. Ten seedlings were planted in each container using a urethane foam block as support. The containers were kept in a control room by maintaining the temperature at 25/20 °C (day/night), with a relative humidity of 60%, a CO_2 concentration of 800 ppm, under a fluorescent light with an intensity of 140~160 μmol/m^2/s and for a photoperiod of 12 hours. The once-used non-renewed solutions (NR) were from the closed hydroponic systems where the lettuce was grown for a period of six weeks and the solution was not changed throughout the growing period. After the lettuce harvest, the NR solution was collected. This bioassay consisted of five types of nutrient solutions, such as the standard 50% 'Enshi' solution, i.e., the new nutrient solution (NNS); the once-used non-renewed solutions (NR); the once-used non-renewed solution treated with ED (NR + ED), the once-used non-renewed solution treated with AC (NR + AC) and the once-used non-renewed solution treated with amberlite XAD-4 (NR + XAD). This bioassay was conducted for two weeks. All the data collected on the growth attributes, yield and chlorophyll content (measured by SPAD, Konica Minolta, Tokyo, Japan) were taken at harvest. Roots samples were also collected at this time for the subsequent analyses of the soluble protein, ROS, lipid peroxidation and antioxidant enzyme activities.

Bioassay Using Twice Used Nutrient Solution (Bioassay II). This bioassay was conducted by using different treatments in the twice-used non-renewed solutions (2NR). The 2NR solutions were collected from the closed hydroponic systems where the lettuce was grown for two times successively and the solution was not changed throughout the two growing periods (12 weeks). The environmental conditions were similar to the first bioassay. This bioassay was composed of six treatments, such as the standard 50% 'Enshi' solution, i.e., the new nutrient solution (NNS), the once-used non-renewed solutions (1NR), the twice-used non-renewed solution (2NR), the twice-used non-renewed solution treated with ED (2NR + ED), the twice-used non-renewed solution treated with AC (2NR + AC) and the twice-used non-renewed solution treated with XAD-4 (2NR + XAD). This bioassay was also conducted for two weeks and the data were taken like in the first bioassay.

Determination of hydrogen peroxide (H_2O_2), superoxide radical ($O_2^{\cdot-}$), and their histochemical detection in roots

The H_2O_2 and $O_2^{\cdot-}$ in the roots were extracted and the content was determined spectrophotometrically as described by Willekens et al. (1995) and Jiang and Zhang (2002), respectively. The H_2O_2 was detected using 3, 3-diaminobenzidine (DAB) staining according to Zeng et al (2014) with some modification. The fresh root tips were incubated in a 1 mg m/L DAB–HCl solution for 8 h and washed once with a 2-N-morpholino-ethanesulfonic acid/potassium chloride (MES/ KCl) buffer (10^{-3} M, pH 6.15). The superoxide anion ($O_2^{\cdot-}$) was detected using nitro blue tetrazolium (NBT) staining (Zeng et al. 2014). The root tip segments were dyed for 2 h with 0.1 mg m/L NBT (in 0.2 M phosphate buffer, pH 7.6) in darkness and subsequently washed once with a phosphate buffer. After staining, the roots were washed with distilled water for 10 min. All the stained segments were observed using a Leica Fluorescence Stereomicroscope (M165C, Leica Microsystems, Heerbrugg, Switzerland) under visible light and photographed with a charge-coupled device (CCD) imaging system, which was fitted to the microscope.

Determination of total soluble protein content in roots

The total soluble protein content was quantified in the lettuce roots using a Spectrophotometric Bradford assay (Bradford 1976). 0.5 g of the fresh roots

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was ground in liquid nitrogen to a fine powder. To avoid protein denaturation, the mortar, the pestle and the Eppendorf tubes were previously frozen in liquid nitrogen. Then, 1.2 mL of the extraction buffer (K-0.2 M phosphate at pH 7.8; 0.1 176 mM EDTA and 1% insoluble PVP) was added to the powder. The samples were vortexed and centrifuged at 4 °C and 15000 g for 30 min. A 5 µL aliquot of the supernatant was carefully collected and mixed with 795 µL of distilled water and 200 µL of the Bradford Bio-Rad (Protein assay) reagent. The absorbance was recorded at a wavelength of 595 nm after 15 to 20 min of reaction using a UV-VIS spectrophotometer (UV-1700 PharmaSpec, Shimadzu Corp., Japan). A calibration curve (0, 2.5, 5, 7.5 and 10 mg/L) was made from the stock solution (20 mg/mL) of the bovine serum albumin (BSA) used as the standard.

Determination of lipid peroxidation in roots

The amount of MDA was assayed to evaluate the effects of the allelochemicals on the rhizosphere of the lettuce. Lipid peroxidation was determined in 0.5 g of the root fresh weight by measuring the amount of MDA, a product of the lipid peroxidation, by the thiobarbituric acid reaction (Gossett et al. 1994). The roots were collected, weighed (0.5 g), immediately frozen in liquid nitrogen and stored at –25 °C until extraction. The frozen tissues were ground with a mortar with pestle, suspended in 0.5 mL of 0.1 mM Tris at pH 8. The extracts were centrifuged at 15 000 rpm for 20 min (4 °C) and the supernatant was used for the MDA determination. The measurement was undertaken at 25 °C using a UV-VIS spectrophotometer (UV-1700 PharmaSpec, Shimadzu Corp., Japan).

Determination of antioxidant enzyme activities in roots

For the determination of the enzyme activities, 0.5 g of the root was homogenized in 8 mL of 50 mM PBS (pH 7.8) using a pre-chilled mortar and pestle, then centrifuged at 15,000 × g for 20 min at 4 °C. The supernatant was designated as a crude enzyme extract and stored at 4 °C for the assays of various antioxidant enzyme activities (Wu et al. 2003). The SOD, POD and CAT activities were determined according to Zhang (1992). The SOD activity was assayed using nitroblue tetrazolium (NBT). The reaction mixture (3 mL) contained 50 mM PBS (pH 7.8), 13 mM methionine, 75 µM NBT, 10 µM EDTA, 2 mM riboflavin, and an enzyme extract (100 µL). The reaction was started by placing the tubes below two 15 W incandescent lamps emitting 4 000 Lux for 15 min and then stopped by switching off the light. The absorbance was measured at 560 nm. One SOD unit was defined as the quantity of the enzyme that produced a 50% inhibition of the NBT reduction under the experimental conditions. The reaction mixture for the POD consisted of 100 µL of enzyme extract, 100 µL of guaiacol (1.5%, v/v), 100 µL of H_2O_2 (300 mM) and 2.7 mL of 25 mM PBK with 2 mM EDTA (pH 7.0). The increase in the absorbance was measured spectrophotometrically at 470 nm (ε = 26.6 Mm/cm). The assay mixture for the CAT contained 100 µL of enzyme extract, 100 µL of H_2O_2 (300 mM) and 2.8 mL of the 50 mM phosphate buffer with 2 mM EDTA (pH 7.0). The CAT activity was assayed by monitoring the decrease in the absorbance at 240 nm as a consequence of the H_2O_2 consumption (ε = 39.4 mM/cm). The APX activity was determined according to Nakano and Asada (1981). The reaction mixture consisted of 100 µL of enzyme extract, 100 µL of ascorbate (7.5 mM), 100 µL of H_2O_2 (300 mM) and 2.7 mL of the 25 mM potassium phosphate buffer with 2 mM EDTA (pH 7.0). The oxidation of the ascorbate was determined by the decrease in the absorbance at 290 nm (ε = 2.8 mM/cm).

Experimental design and statistical analysis

All the experiments were arranged in a completely randomised design with three replications. An analysis of variance (ANOVA) for all the data was undertaken using the computer package MSTAT-C developed by Russel (1986). The mean differences were separated according to Tukey's test at $P < 0.05$.

RESULTS

Bioassay I

The lettuce seedlings grown in the NR solution showed retarded growth in all the studied parameters (Table 1). The lowest shoot and root dry weight were observed in the NR solution plants. While the shoot and root dry weight were the highest in the plants grown in the NNS solution. When the ED, XAD and AC treatments were applied to the NR solution, the seedling growth improved. The NR + AC, NR + ED and NR + XAD solutions plants produced a shoot and root dry weight similar to the NNS solution plants. The other growth parameters, such as the number of leaves/plant, the maximum leaf

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Table 1. Influence of different treatments to the once used nutrient solution on the growth of lettuce seedlings (Bioassay I)

Types of nutrient solutions	No. of leaves /plant	Max. leaf length (cm)	Max. leaf width (cm)	Longest root length (cm)	Shoot fresh weight/plant (g)	SPAD	Dry weight/plant (g)	
							shoot	root
NNS[z]	10.3[au]	14.3[a]	7.7[a]	22.1[a]	13.1[a]	36.3[a]	0.67[a]	0.075[a]
NR[y]	8.6[b]	10.1[b]	5.6[b]	15.9[b]	10.2[b]	33.6[b]	0.51[b]	0.059[b]
NR + ED[x]	10.2[a]	14.2[a]	7.6[a]	21.4[a]	12.9[a]	35.9[a]	0.66[a]	0.076[a]
NR + XAD[w]	9.5[a]	13.9[a]	7.2[a]	20.1[a]	12.3[a]	34.6[a]	0.61[a]	0.071[a]
NR + AC[v]	9.8[a]	13.4[a]	7.1[a]	20.3[a]	11.9[a]	34.9[a]	0.62[a]	0.072[a]

[z]New nutrient solution i.e. Standard 50% 'Enshi' solution; [y]once used non-renewed nutrient solutions where lettuce was grown for a period of 6 weeks and the solution was not changed throughout the growing period; [x]NR solution treated with electro-degradation; [w]NR solution treated with amberlite XAD-4; [v]NR solution treated with activated charcoal; [u]means within a column for each bioassay followed by different letters are significantly different at $P < 0.05$

length and width, the longest root length, the SPAD (Soil Plant Analysis Development) value and shoot fresh weight followed a similar trend.

The H_2O_2 and $O^{\cdot-}$ generation in the plant roots grown in the different types of nutrient solutions varied significantly (Figure 1A, B). The highest H_2O_2 and $O_2^{\cdot-}$ production was observed in the roots of the NR solution plants. The intense staining of the NR solution plant roots by DAB (Figure 2A) and by NBT (Figure 2B) were observed which also indicated the higher accumulation of H_2O_2 and $O_2^{\cdot-}$, respectively. The lowest H_2O_2 and $O_2^{\bullet-}$ generation was observed in the NNS solution plant roots. The plants grown in the NR+ED solution generated H_2O_2 and $O_2^{\cdot-}$ in their roots similar to the plants in the NNS solution. However, the plants roots grown in the NR+AC and NR+XAD solutions generated higher H_2O_2 and $O_2^{\cdot-}$ compared to the plant roots in the NNS solution. The MDA accumulation in the lettuce roots also varied significantly in the plants grown in the different types of nutrient solutions (Figure 1C). The highest MDA accumulation was observed in the plants' roots from the NR solution. The plants grown in the NR + ED and NNS solution accumulated statistically similar amount of MDA in their roots. The MDA accumulation in the plants' roots grown in the NR + AC and NR + XAD solutions were moderately high compared to the MDA accumulation in the plant roots in the NNS solution. The soluble protein content was also highest in the plant roots in the NR solution (Figure 1D) while it was lowest in the plant roots in the NR + ED and NNS solution. The plant roots in the NR + AC and NR + XAD solution produced a relatively high amount of soluble protein compared to the plants grown in the NR + ED and NNS solution.

The antioxidant enzyme activity was significantly diverse in the plant roots grown in the various types of nutrient solutions (Table 2). The SOD activity was relatively higher in the plant roots grown in the NNS and NR + ED solution, but it was relatively lower in the plant roots of the NR, NR + AC and NR + XAD solution. Similarly, the POD activity was relatively higher in the plants grown in the NNS, NR + ED and also in the NR + XAD solution, but

Table 2. Influence of different treatments to the once used nutrient solution on the antioxidant enzymes activity in the roots of lettuce seedlings (Bioassay I)

Types of nutrient solutions	SOD (U/g FW)	POD (mmol/g FW/min)	CAT (mmol/g FW/min)	APX (mmol/g FW/min)
NNS[z]	71.52 [a]	5.72 [a]	0.82 [a]	23.14 [a]
NR[y]	38.11 [b]	4.38 [b]	0.56 [b]	14.81 [b]
NR + ED[x]	61.21 [a]	6.78 [a]	0.81 [a]	21.75 [a]
NR + XAD[w]	48.97 [b]	5.99 [a]	0.93 [a]	19.56 [a]
NR + AC[v]	42.17 [b]	4.78 [b]	0.91 [a]	20.67 [a]

SOD – superoxide dismutase; CAT – catalase; POD – guaiacol peroxidase; APX – ascorbate peroxidase; for other abbreviations see Table 1

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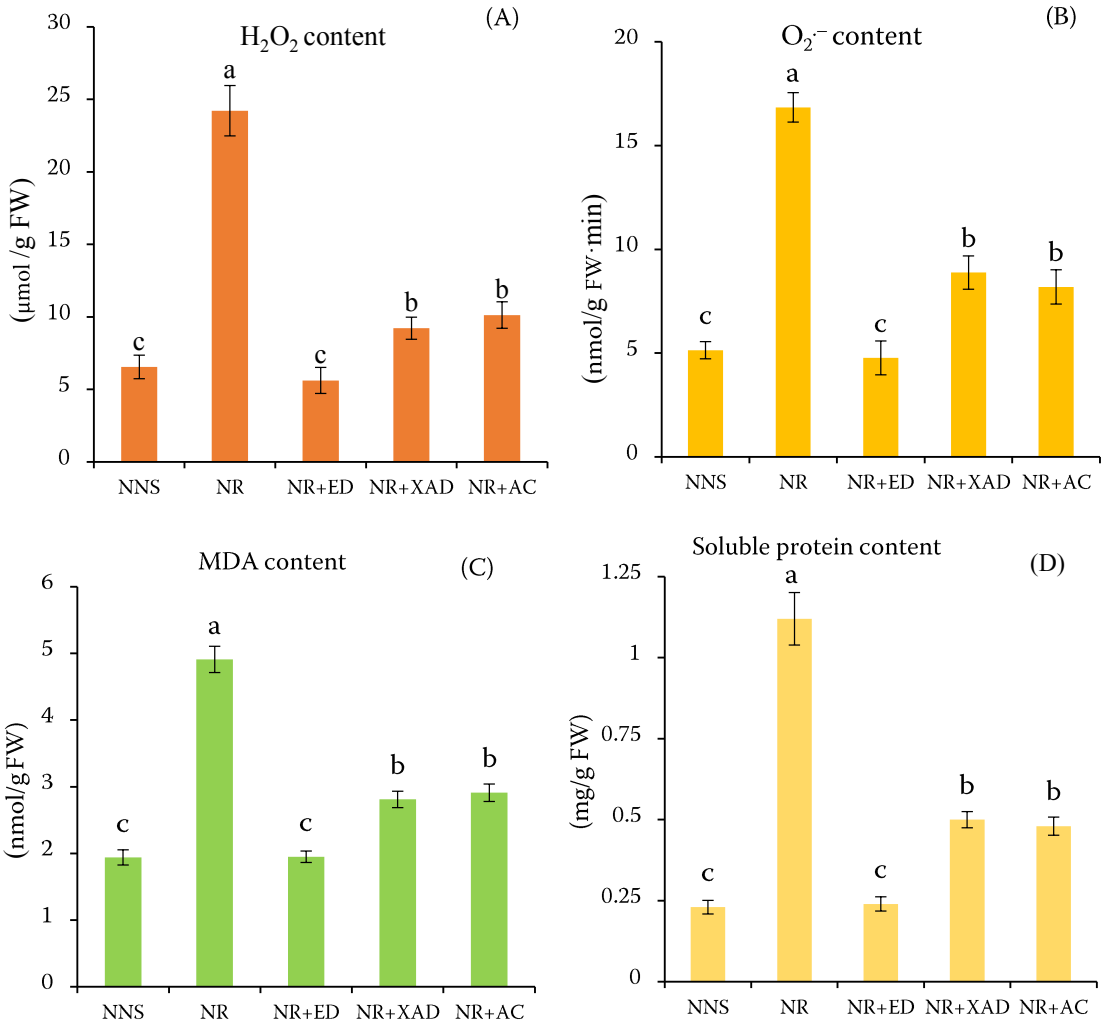

Figure 1. Effect of once used non-renewed nutrient solution and it's treatment by different methods on the generation of (A) H_2O_2, (B) $O_2^{\cdot-}$, (C) MDA, and (D) soluble protein in the lettuce roots

The vertical bars represent SE (n = 3); different letters above each bar are significant according to the Tukey's multiple range test at $P < 0.05$; NNS – new nutrient solution; NR – once used non-renewed solution; NR + ED – NR solution treated with ED; NR + XAD – NR solution treated with XAD-4; NR + AC – NR solution treated with AC; MDA – malondialdehyde

it was relatively lower in the roots of the NR and NR + AC solution plants. On the other hand, both the CAT and APX activity were lower in the plants grown in the NR solution. The plants grown in the NNS, NR + ED, NR + AC and also NR + XAD solution showed a statistically similar CAT and APX activity in their roots.

Bioassay II

The growth of the lettuce seedlings was notably varied due to the influence of the 2NR solution treatments (Table 3). The lowest shoot and root dry weight were observed in the 2NR solution plants while these shoot and root dry weight were the highest in the NNS solution plants. The ED, XAD and AC application to the 2NR solution also improved the seedling growth. The plants grown in the 2NR + AC, 2NR + ED and 2NR + XAD solutions produced a shoot and root dry weight similar to the plants in NNS solution. The shoot and root dry weights of the plants in 1NR solution were higher than the 2NR solution plants, but lower than the plants grown in all the other solutions. The other growth parameters, such as the number of leaves/plant, the maximum leaf length and width, the longest root length, the SPAD value and the shoot fresh weight followed an almost similar trend.

The H_2O_2 and $O_2^{\cdot-}$ generation in the plant roots grown in the different types of nutrient solutions varied significantly (Figure 3A, B). The highest H_2O_2

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Table 3. Influence of different treatments to the twice used nutrient solution on the growth of lettuce seedlings (Bioassay II)

Types of nutrient solutions	No of leaves per plant	Max. leaf length (cm)	Max. leaf width (cm)	Longest root length (cm)	Shoot fresh weight/plant (g)	SPAD	Dry weight/plant (g)	
							shoot	root
NNS[z]	11.1[at]	15.2[a]	8.0[a]	20.9[a]	15.10[a]	35.8[a]	0.64[a]	0.076[a]
1NR[y]	8.6[b]	9.9[b]	5.8[b]	16.3[b]	11.15[b]	33.9[b]	0.54b	0.065[b]
2NR[x]	8.4[b]	9.8[b]	5.6[b]	11.8[c]	8.14[c]	33.9[b]	0.48[c]	0.052[c]
2NR + ED[w]	10.6[a]	13.9[a]	7.8[a]	20.9[a]	13.94[a]	35.7[a]	0.65[a]	0.075[a]
2NR + XAD[v]	10.4[a]	13.6[a]	6.9[a]	19.9[a]	13.63[a]	34.5[a]	0.61[a]	0.072[a]
2NR + AC[u]	10.2[a]	13.4[a]	7.3[a]	19.4[a]	13.14[a]	34.8[a]	0.62[a]	0.072[a]

For abbreviations see Table 1

and $O_2^{\cdot-}$ production was observed in the plant roots from the 2NR solution, followed by the plants in the 1NR solution. At this time, the intense staining of the roots from the 1NR and 2NR solution by DAB (Figure 4A) and by NBT (Figure 4B) was also observed, which also indicated a higher accumulation of H_2O_2 and $O_2^{\cdot-}$, respectively. The lowest H_2O_2 and $O_2^{\cdot-}$ generation was observed in the plant roots from the NNS solution which was statistically the same to the plants in the 2NR + ED solution. However, the 2NR + AC and 2NR + XAD solutions plant roots generated higher H_2O_2 and $O_2^{\cdot-}$ compared to the plants in the NNS and 2NR + ED solution.

The MDA accumulation in the lettuce roots also varied significantly in the plant roots grown in the different types of nutrient solutions (Figure 3C). The highest MDA content was observed in the plant roots from the 2NR solution followed by the plants in the 1NR solution. The plants grown in the 2NR + ED and NNS solution accumulated statistically the same MDA in their roots. The MDA accumulation in the plants from the 2NR + AC and 2NR + XAD solutions was higher than the MDA accumulation in the plants from the 2NR + ED and NNS solution. The soluble protein content was also the highest in the plant roots from the 2NR solution followed

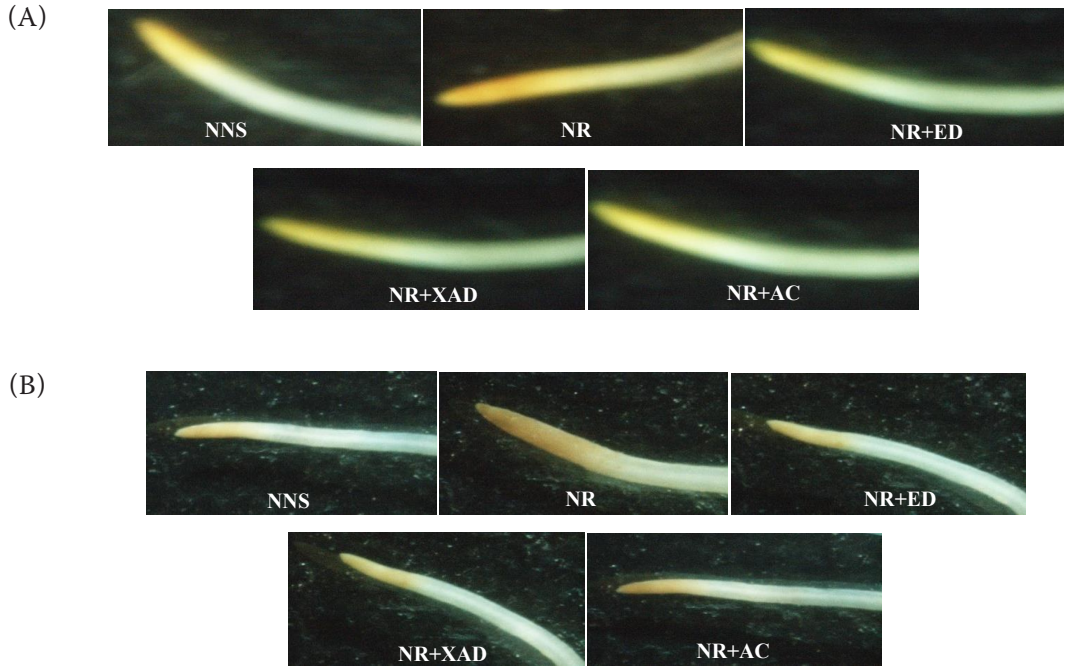

Figure 2. Histochemical detection of (A) H_2O_2 and (B) $O_2^{\cdot-}$ in lettuce root grown in Bioassay I. Stained root was observed at ×60 using a Leica Fluorescence Stereomicroscope (M165C, Leica Microsystems, Heerbrugg, Switzerland) under visible light and photographed with a charge-coupled device (CCD) imaging system

For abbreviations see Figure 1

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Table 4. Influence of different treatments to the twice used nutrient solution on the antioxidant enzymes activity in the roots of lettuce seedlings (Bioassay II)

Types of nutrient solutions	SOD (U/g FW)	POD (mmol/g FW/min)	CAT (mmol/g FW/min)	APX (mmol/g FW/min)
NNS[z]	60.11[a]	6.98[a]	0.84[a]	23.61[a]
1NR[y]	49.39[b]	3.99[b]	0.71[b]	17.51[b]
2NR[x]	41.38[c]	3.25[c]	0.47[c]	11.94[c]
2NR + ED[w]	56.38[a]	6.97[a]	0.80[a]	21.82[a]
2NR + XAD[v]	47.18[b]	4.12[b]	0.74[a]	22.71[a]
2NR + AC[u]	46.91[b]	4.15[b]	0.78[a]	22.63[a]

SOD – superoxide dismutase; CAT – catalase; POD – guaiacol peroxidase; APX – ascorbate peroxidase; for other abbreviations see Table 1

by the 1NR solution, while it was the lowest in the plants from the 2NR + ED and NNS solution (Figure 4D). The plants grown in the 2NR + AC and 2NR + XAD solution produced the statistically same and a relatively high amount of the soluble protein compared to the plants root in the 2NR + ED and NNS solution.

The antioxidant enzyme activity was considerably different in the plant roots grown in the various types of nutrient solutions (Table 4). The SOD activity was the highest in the plants grown in the NNS and 2NR + ED solution followed by plants in the 1NR, 2NR + AC and 2NR + XAD solution, but it was the lowest in the roots of the plants in 2NR solution. Similarly, the

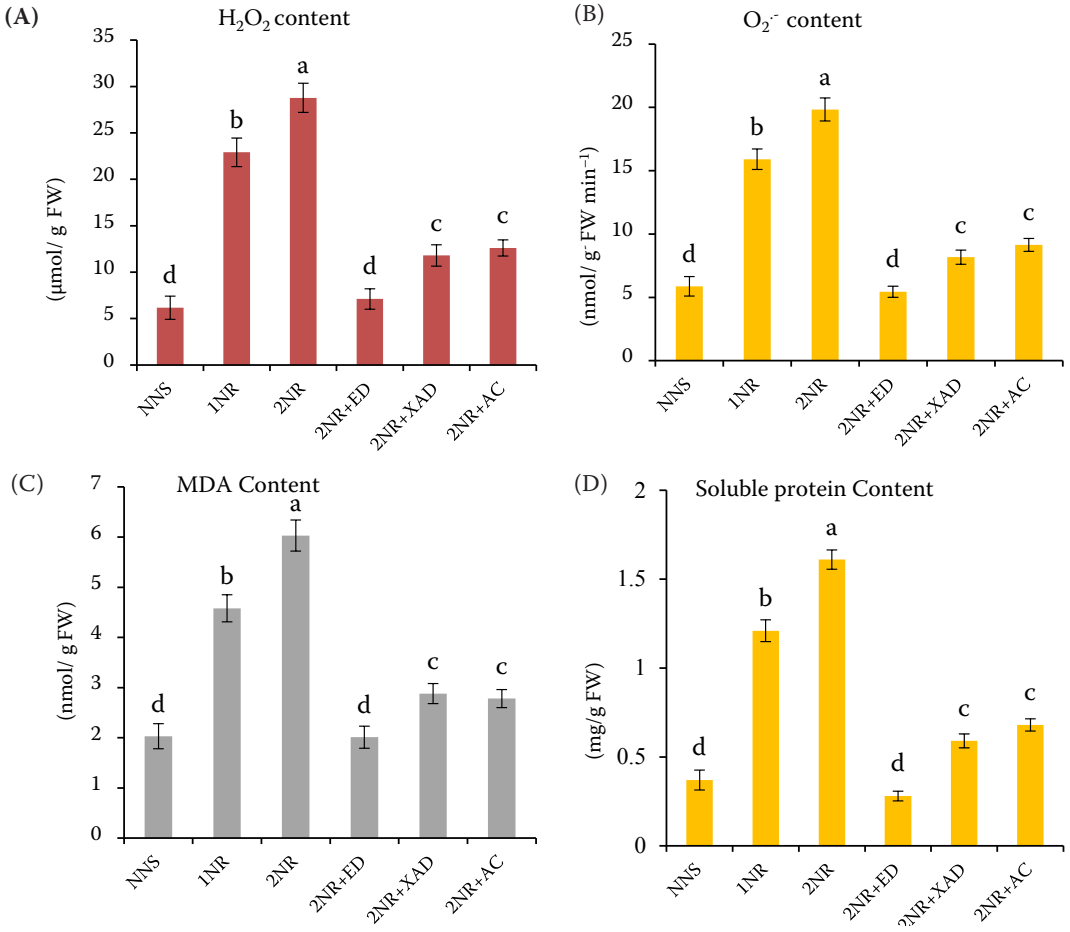

Figure 3. Effect of twice used non-renewed nutrient solution and it's treatment by different methods on the generation of (A) H_2O_2, (B) $O_2^{\cdot-}$, (C) MDA and (D) Soluble protein in the lettuce roots

The vertical bars represent SE (n = 3); for abbreviations see Figure 1

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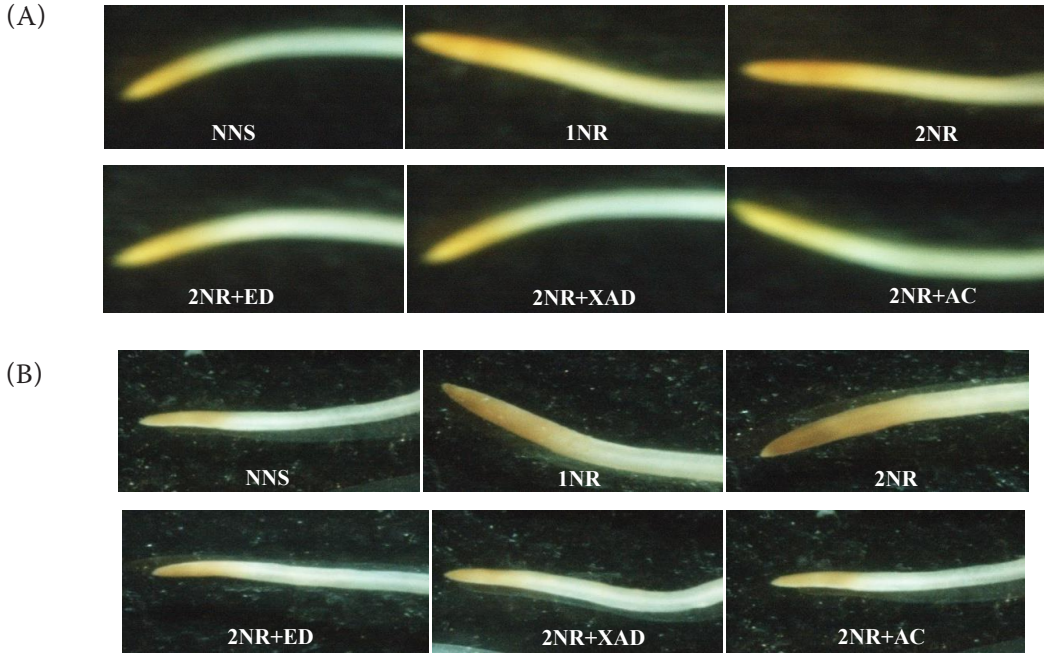

Figure 4. Histochemical detection of (A) H_2O_2 and (B) $O_2^{\cdot-}$ in lettuce root grown in Bioassay II. Stained root was observed at ×60 using a Leica Fluorescence Stereomicroscope (M165C, Leica Microsystems, Heerbrugg, Switzerland) under visible light and photographed with a charge-coupled device (CCD) imaging system

POD activity was also the highest in the plants grown in the NNS and 2NR + ED solution followed by the plants in the 1NR, 2NR + AC and 2NR + XAD solution and it was the lowest in the roots of the plants in the 2NR solution. Likewise, both the CAT and APX activity in the plants root from the NNS, 2NR + ED, 2NR + AC and 2NR + XAD solution were statistically the same and they were the lowest in the roots from the 2NR solution plants. The plants grown in the 1NR solution showed a relatively high CAT and APX activity compared to the plants in the 2NR solution.

DISCUSSION

Closed hydroponic systems have a problem of the allelochemical accumulation in the culture solution. Many researchers found allelochemicals in the culture solution from the root exudation causing allelochemical stress in the strawberry (Kitazawa et al. 2005; Asao et al. 2008; Asaduzzaman et al. 2012; Mondal et al. 2013; Talukder et al. 2018), the tomato (Yu, Matsui 1993), cucumber (Yu, Matsui 1994; Asao et al. 1998), several leafy vegetables (Asao et al. 2004b) and some ornamentals plants (Asao et al. 2007) grown in closed hydroponics. Lettuce grown in closed hydroponic accumulates many allelochemicals in the culture solutions (Asao et al. 2004b).

Currently, some research findings detected many organic acids, such as benzoic, phenyl acetic, cinnamic, p-hydroxybenzoic, lauric, phthalic, vanillic, palmitic, and stearic acids etc. from the lettuce root exudates grown in the non-renewed nutrient solution of closed hydroponics (Asao et al. 2004b; Lee et al. 2006) and identified them as major growth inhibitors. These accumulated allelochemicals create an allelochemical stress on the plants. In our present study, we tried to analyse the effects of the allelochemical stress on the lettuce roots on a biochemical and molecular level, particularly on the activities of the antioxidant enzymes, such as CAT, APX, SOD and POD and the generation of H_2O_2, $O_2^{\cdot-}$, the soluble protein and MDA.

In our first bioassay, all the growth parameters, such as the leaf number/plant, leaf size, root length, shoot fresh weight, shoot and root dry weight, etc. were significantly reduced in plants grown in the NR solution (Table 1). We earlier described that several allelochemicals accumulated in the reused solution for the lettuce culture. These allelochemicals were delivered into the rhizosphere due to the root exudation were found to be responsible for hampering numerous physiological process (Bertin et al. 2003; Inderjit, Duke 2003; Blum 2005). Therefore, we obtained a reduced seedling growth in the NR solution.

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Compared to the plants grown in the NNS solution, the production of ROS (H_2O_2 and $O_2^{\cdot-}$) was higher in the NR solution plants (Figures 1A, B and 2A, B). The generation of ROS is one of the earliest biochemical responses of eukaryotic cells to biotic and abiotic stresses (Apel, Hirt 2004). The production of ROS in plants acts as a secondary messenger to trigger subsequent defence reactions in the plants. The most common ROS are hydrogen peroxide, superoxide, the hydroxyl radical, and the singlet oxygen that formed as a natural by-product of the normal metabolism of oxygen and is crucial in cell signalling. The overproduction of ROS leads to oxidative stress and can cause damage to the cellular components. Plants generate more ROS when exposed to stressful conditions (Yamamoto et al. 2003; Halliwell 2006; Rhoads et al. 2006). Allelopathic compounds also induce an oxidative stress manifested as an enlarged production of the toxic ROS (Cruz-Ortega et al. 2002; Bais et al. 2003; Weir et al. 2004; Sánchez-Moreiras et al. 2005; Batish et al. 2006; Singh et al. 2006). Several other studies have also shown that allelochemical stress can cause oxidative damage, which indicated the enhanced production of ROS (Baziramakenga et al. 1995; Politycka 1996; Yu et al. 2003; Lara-Nunez et al. 2006; Ye et al. 2004, 2006). Cruz-Ortega et al. (2007) stated that allelochemical stress not only induced an oxidative damage responsible for toxicity of the plant by generating ROS, but they also believed that the generated ROS might only act as signals activating cascade of other events leading to cell malformations. On the other hand, Foreman et al. (2003) described that the overproduction of ROS showed a decline in the activity of the NADPH oxidase enzyme that was known to enhance the root development and elongation. Since many allelochemicals accumulated in the NR solution, we observed a higher production of ROS (H_2O_2 and $O_2^{\cdot-}$) in the roots of the plants grown there. This over-accumulation of ROS was not sufficiently scavenged by the antioxidant enzymatic system and was responsible for damage to the cellular components and ultimately caused cell death (Oracz et al. 2007). That is why, we obtained a reduced root growth in the NR solution plants which led to a lower water and nutrients uptake. Consequently, retarded plant growth was obtained in the NR solution plants.

Oxidative damage is quantified by measuring the MDA content. The high level of MDA in plants grown in the NR solution subjected to the allelochemical stress indicated that the antioxidant enzymatic system did not completely eliminate the over-generated ROS (Figure 1C). It revealed that the plants grown in the NR solution suffered much from oxidative damage. Qian et al. (2009) found similar results in *Chlorella vulgaris*. They observed that plants exposed to allelochemical stress experienced oxidative damage and produced a high level of MDA. Some other reports showed that allelochemical stress induced oxidative damage evidenced by a high level of lipid peroxidation (Romero-Romero et al. 2005; Lara-Núñez et al. 2006).

We also obtained a higher amount of the soluble protein in the plants grown in the NR solution (Figure D) compared to the plants in the NNS solution. It is well known that plants under stress conditions may accumulate a small molecular mass soluble protein that could be used as a source of storage of nitrogen and can be rapidly mobilised when required for the alleviation of stress (Singh et al. 1987), and additionally, these proteins could also have a role in the osmotic adjustment (Ashraf et al. 2004). A higher amount of soluble protein in the NR solution plant roots was a sign of the stress condition induced by the allelochemicals. Ahmed et al. (2013) also found a higher production of the soluble protein under stress conditions in barley. Collectively, the excess production of the ROS, MDA and soluble protein in plants grown in the NR solution were observed compared to the NNS solution plants. Therefore, it is evident that plants grown in the NR solution suffered more from the allelochemical stress and accordingly had more excessive oxidative damage and lipid peroxidation.

To minimise the impact of the allelochemical induced oxidative stress, plants have evolved a complex system of enzymatic antioxidants, SOD, CAT, POD, GR, and APX, and non-enzymatic antioxidants, ascorbic acid, α-tocopherol, reduced glutathione, ß-carotene, polyamines (PAs), salicylates, compatible solutes, such as proline (Pro), glycine betaine (GB), and zeaxanthin that accumulate in higher amounts in plants under stress conditions (Ozkur et al. 2009). Plants boost up the production of antioxidants in order to minimise the detrimental effects of the oxidative stress to normalise their metabolic activities under oxidative stress. Different antioxidants have different roles in protecting cells in specific compartments and in particular conditions. It is generally recognised that $O_2^{\cdot-}$ might be converted to H_2O_2 and then metabolised to water by APX and GR in plants to maintain membrane structures (Foyer, Fletcher 2001). Similarly, several other antioxidant

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enzyme molecules are responsible for counteracting the deleterious effects of ROS. Initially, SOD catalyses the conversion of $O_2^{\cdot-}$ to H_2O_2, which is further reduced to water by APX by using ascorbate as an electron donor (Scandalios 2005). CAT and POD are involved in the degradation of hydrogen peroxide into water and oxygen to prevent the oxidative damage (Willekens et al. 1995; Mittler 2002). The elevated accumulation of antioxidant enzymes, such as SOD, CAT, GR, APX, and POD, is involved in lowering the oxidative injury that was observed in caper bush seedlings under drought stress (Ozkur et al. 2009). Similarly, Yang et al. (2009) found an increase in the activity of CAT, SOD, POD, APX, and GR under stress conditions.

On the other hand, in our present study, the antioxidant enzyme activity, such as SOD, POD, CAT and APX, were significantly lower in the plant roots grown under allelochemical stress in the NR solutions (Table 2). These results agree with other studies that have described antioxidant enzyme activity under allelochemical stress. It has been reported that secalonic acid, isolated from *Aspergillus japonicus*, significantly reduced the SOD and POD activity in seeds of rape, cucumbers, corn and sorghum (Zeng et al. 2001). Likewise, aqueous extracts from rice reduced the SOD activity in barnyard grass (Lin et. al. 2000). Recently, Sánchez-Moreiras and Reigosa (2005) have shown that 2(3H)-Benzoxazolinone (BOA) severely inhibited the SOD activity in the leaves and roots of lettuce.

Dorning and Cipollini (2006) described that the effect of allelochemicals on the plant antioxidant enzymes system are dosage-dependent. Yan et al. (2015) found that the antioxidant enzyme activity showed an increasing trend at low concentrations, followed by a decline phase at high concentrations in patchouli seedlings. Qian et al. (2009) stated that N-phenyl-2-naphthylamine was an allelochemical of the unicellular green alga *Chlorella vulgaris* and found that the activities of SOD and POD increased in lower (2.5 mg/L) concentrations and decreased at higher (4 mg/L) concentrations of N-phenyl-2-naphthylamine. Berberine is also known to act as an allelochemical in aquatic ecosystems as it inhibits the growth of the cyanobacteria *Microcystis aeruginosa* (Zhang et al. 2011). It up-regulated the SOD activity at lower concentrations while down-regulated the activity at higher concentrations. A more detailed study has been done on ethyl 2-methylacetoacetate (EMA), an allelochemical of *Chlorella pyrenoidosa* and *M. aeruginosa* (Li, Hu 2005). These plants that responded to EMA at lower concentrations showed an increased activity of SOD and POD. However, higher concentrations of EMA led to a decreased activity of the enzymes. Similar trends of SOD, POD, CAT and APX activities were also obtained in the cucumber after a cinnamic acid treatment (Ding et al. 2007). Therefore, these findings supported our results that higher concentration levels of allelochemicals in the NR solution resulted in the declined antioxidant enzyme (SOD, POD, CAT and APX) activity and ultimately resulted in a potent inhibitory effect on the plant growth.

Some other researchers explained that the antioxidant enzyme activities in plants caused by allelochemical stress were not only limited to the allelochemical concentrations, but also dependent to the allelochemical exposure duration. The CAT activity in *M. aerunginosa* cells treated with EMA showed the highest concentration after the dissolution of a medium concentration of the allelochemical (1 mg/L) and generally declined upon increasing the concentration with a longer than 2 day exposure to the allelochemical (Hong et al. 2008). Zhang et al. (2011) also stated that the antioxidant enzyme activities in plants depended on the duration of the allelochemical treatment. In our experiments, the lettuce seedlings were grown in the NR solution for 2 weeks. So, we speculated that the seedlings were exposed to the allelochemicals in the NR solution for a longer period and thus, reduced the antioxidant enzyme activity, leaving the plant under the risk of oxidative damage. Therefore, the allelochemical stress in the NR solution plants were specified by producing an oxidative imbalance evidenced by the overaccumulation of ROS and the reduction of the antioxidant enzyme activity in the roots.

It was previously well established that plants grown in an NR solution were exposed to allelochemical stress. For that reason, the solution has to be eventually renewed to grow crops free from autotoxic conditions. However, recently the disposal of the culture solution has been discouraged, due to the environmental pollution. For the effective removal or degradation of the allelochemical from the nutrient solution and consequently, to increase the crop yield, several researchers have used several methods in different crops. For instance, the degradation of the allelochemicals in the strawberry production by ED means (Asao et al. 2008; Asaduzzaman et al. 2012; Talukder et al. 2019a,b), the adsorption of the

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allelochemicals in the lettuce production by AC and XAD-4 (Lee et al. 2006), the degradation of the allelochemicals in the cucumber production by microbial strains (Asao et al. 2004a).

In our study, we applied the ED, AC and XAD-4 method to alleviate the allelochemical stress in the first bioassay. Due to the ED application, we obtained a growth (Table 1), ROS, MDA and soluble protein production (Figure 1) and antioxidant enzyme activities (Table 2) in the plants grown in the NR + ED solution that were similar to the plants in the NNS solution. When the AC and XAD-4 method were applied, we obtained a plant growth in the NR + AC and NR + XAD solution similar to the plants in the NNS solution, but those plants generated more ROS, MDA and soluble protein compared to the plants in the NNS solution. We also obtained a lower SOD activity in the plants in the NR + AC and NR + XAD solution and a lower POD activity in the plants from the NR + AC solution. From these results, we speculated that the ED method degraded the allelochemicals from the NR solution more efficiently, whereas the AC and XAD-4 method did not absolutely adsorb the allelochemicals.

In the second bioassay, compared to the NNS solution plants, all the growth parameters were lower (Table 3), the MDA, ROS and soluble protein content in the roots were higher (Figure 3) and the antioxidant enzyme activities in the roots were lower (Table 4) in the plants from the 1NR solution. On the other hand, compared to the 1NR solution plants, all the growth parameters were lower and the MDA, ROS and soluble protein content in the roots were higher and the antioxidant enzyme activities in the roots were lower in the 2NR solution plants. Thus, the plants in the 2NR solution suffered from more oxidative damage and consequently, more retarded growth was observed in the 2NR solution plants. This might be due to the higher concentration of the allelochemicals in the 2NR solution. As the 2NR solution was used for a longer period for the cultivation of the lettuce compared to the 1NR solution, a higher amount of the allelochemicals accumulated there. Therefore, the plants grown in the 2NR solution suffered the most from the allelochemical stress. These results were also supported by the findings of other researchers. Lee et al. (2006) determined that the amount and concentration of allelochemicals in the nutrient solution highly varied with reuse time, generally showing an increasing trend with an increased reuse time. They found that a few allelochemicals were exuded from the roots at a comparatively low concentration in the 1NR solution and, later on, the amount of the allelochemicals and their concentrations were found to be increased in the 2NR solution. As the amount of allelochemicals were found to be increased in the 2NR solution, they badly affected the plant growth by an additive or synergistic means (Inderjit 1996). As a result, in our present study, the most retarded growth in the lettuce was obtained in the 2NR solution.

Similar to the first bioassay, when the ED was applied, we obtained a plant growth, ROS, MDA and soluble protein production and antioxidant enzyme activities in the plant roots from the 2NR + ED solution that were similar to the plants from the NNS solution. When the AC and XAD-4 method were applied, we also obtained plant growth in the 2NR + AC and 2NR + XAD solution plants similar to the NNS solution plants, but those plants generated more ROS, MDA and soluble protein compared to the NNS solution plants. At this time, we also obtained a lower SOD and POD activity in plants from both in the 2NR + AC and 2NR + XAD solution. These results indicate that the AC and XAD-4 applied to the 1NR and 2NR solution exposed the plants to a little more oxidative imbalance compared to the NNS solution plants.

CONCLUSION

Plants grown in the 1NR solution are exposed to allelochemical stress as evidenced by the generation of more ROS, MDA and soluble protein and lower antioxidant enzyme activity in the roots. As a result, oxidative damage occurred in the roots that hamper the water and nutrients uptake in the plants. Ultimately, we obtained retarded growth in the 1NR solution plants. By the same mechanisms, the oxidative damage and retarded growth were more pronounced in the 2NR solution plants. The ED to 1NR and 2NR solution maintained the plant growth and oxidative balance like the NNS solution plants.

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