Journal of Plant Growth Regulation
https://doi.org/10.1007/s00344-020-10239-5

Effects of Olive Mill Wastewater and Two Natural Extracts as Nitrification Inhibitors on Activity of Nitrifying Bacteria, Soil Nitrate Leaching Loss, and Nitrogen Metabolism of Celery (*Apium graveolens* L.)

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Received: 7 February 2020 / Accepted: 29 September 2020

Abstract
Minimizing nitrification of fertilizer ammonium (NH_4^+) can reduce nitrate (NO_3^-) contamination of groundwater and increase nitrogen (N) use efficiency (NUE). Olive mill wastewater (OMW), hydroalcoholic extracts of *Mentha piperita* L. (Mp) and *Artemisia annua* L. (Aa), and synthetic nitrification inhibitor (NI) dicyandiamide (DCD) were investigated. All NIs reduced activity of nitrifying bacteria and NO_3^- leached and increased efficiency of N metabolism of celery (*Apium graveolens* L.) during 56 days in a soil mixture fertilized with ammonium sulfate [$(NH_4)_2SO_4$]. Soil NO_3^- leaching losses of DCD, OMW, Mp, and Aa treatments were 24%, 26%, 67%, and 78% of the untreated control loss, respectively, at 35 and 56 days after planting (DAP). Decreased nitrification by NIs resulted in greater concentrations of soil NH_4^+ correlated with less nitrate reductase (NR) activity in roots and leaves and less soil acidification compared to the control. At 35 and 56 DAP, DCD and OMW treatments decreased NR and increased glutamine synthetase activities in leaves and roots, compared to the control. NIs increased leaf and root protein and amino acids. OMW significantly decreased leaching loss of NO_3^- to 10% of fertilizer N applied to the soil, compared to 38% of applied NH_4^+-N leached from the control. OMW proved an effective alternative to DCD to improve NUE of NH_4^+-fertilizers.

Keywords *Mentha piperita* L. · *Artemisia annua* L. · Dicyandiamide · Ammonium · Nitrite · Amino acids

Introduction

Large amounts of ammoniacal fertilizers commonly used to increase crop yield and quality (Sutton et al. 2011) can disrupt both small- and large-scale ecosystems (Smil 2011). Despite extensive fertilization, nitrogen use efficiency (NUE) is often sub-optimal in intensive crop production systems (Raun and Johnson 1999; Knudsen et al. 2006; Vitousek et al. 2009). High application rates of fertilizers supplying N as urea, NH_4^+ , or NO_3^- to crops have been associated with N loss from soil in the liquid (NO_3^- leaching) and gas (N_2O emission) phases, resulting in increasing environmental problems (Glass 2003; Ishikawa et al. 2003; Zhu et al. 2003; Schröder 2014; Qu et al. 2014). It has been estimated that when 50% of the N applied to the field in the form of NH_4^+ or urea is assimilated by the plants, about 50% is lost as NO_3^- by leaching and by denitrification (Flores et al. 2005; Schlesinger 2009). Loss of N from the rhizosphere is a significant economic problem, in addition to unknown costs of NO_3^- pollution of groundwater, eutrophication of surface waters, and atmospheric pollution (Giles 2005; Galloway et al. 2008; Hoang and Alauddin 2010). In southern Italy, 20% of agricultural soils monitored in 2015 lost more than 100 mg NO_3^- kg^{-1} (Colombo et al. 2015), and samples of water in more than 85% of European agricultural areas (about 90 million ha) have NO_3^- levels greater than the freshwater threshold limit of 25 mg L^{-1} (1991 EU Nitrates Directive; Arauzo and Valladolid 2013).

Nitrosomonas spp. and *Nitrosococcus* spp. perform the oxidation of NH_3 to NO_2^- and *Nitrobacter* spp. and

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Published online: 17 October 2020

Nitrospira spp. complete nitrification by the oxidation of NO_2^- to NO_3^-, as below:

$$NH_3 + O_2 \rightarrow NO_2^- + 3H^+ + 2e^-$$

$$NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^-$$

Acidification of the soil matrix, due to the extrusion of protons in low-buffered soils and soilless media can significantly modify the soil microbiome and have a deleterious impact on crop growth (Cytryn et al. 2012). Such modifications of the soil environment not only affect crop growth and the production of NO_2^- and nitrate, but can also select a different community of nitrifying microorganisms best adapted to these new conditions. Nitrification can occur in extreme environments that pure cultures of nitrifiers cannot tolerate (Bock et al. 1986).

The regulation of microbial nitrification processes influences soil N recovery by crops and can increase agronomic NUE (Barneze et al. 2015). Nitrification is a key factor that determines the forms of N present in soil and, therefore, how N is assimilated (Leininger et al. 2006). The oxidation state of N in the soil is a very important factor influencing plant productivity and environmental quality, and the oxidation states of N in NO_3^- and NH_4^+, the two major forms of N that plants can take up by their roots systems, are + 5 and − 3, respectively (Marschner et al. 1996; Marschner 2002). Nitrate can be easily absorbed by plant roots and microbes, and it is also very soluble and mobile in soil. Ammonium, with its positive charge, however, is less subject to loss by leaching because it is easily adsorbed by negatively charged soil colloids such as soil clay minerals and by functional groups of soil organic matter (Mengel and Rehm 2000; Nommik and Vahtras 1982).

Synthetic compounds, such as DCD and 3,4-dimethylpyrazole phosphate (DMPP), inhibit nitrification and improve N recovery—as secondary effects—by increasing the NH_4^+ adsorbed in soil and decreasing leaching losses of NO_3^- from the root zone (Prasad and Power 1995; Panàkovà et al. 2016). These synthetic NIs can decrease the microbial oxidation rate of NH_4^+ by decreasing the activity of bacteria belonging to the genus *Nitrosomonas* (Serna et al. 2000; Kleineidam et al. 2011). DCD, compared to DMPP, is highly soluble, and intensive atmospheric precipitation can lead to the translocation of DCD within the soil profile, reducing its efficiency (Barth et al. 2001; Zerulla et al. 2001; Irigoyen et al. 2003; Fangueiro et al. 2009; Cahalan et al. 2015).

Inhibition of nitrification using eco-friendly and biodegradable plant extracts has been proposed as a promising alternative to a chemical NI (Opoku et al. 2014). Plants have been observed to inhibit nitrification in some ecosystems, and inhibition of nitrification caused by plants in ecosystems has also been hypothesized as a major driving force for the development of low-NO_3^- ecosystems (Lodhi 1978; McCarty 1999; Krishnapillai 1979; Lata et al. 2004). Oils of *Mentha spicata* L. and *A. annua* L. have been shown to inhibit nitrification (Kiran and Patra 2003).

Secondary metabolites with antimicrobial properties in essential oils of *M. piperita* L. (Mp) and *A. annua* L. (Aa) have decreased the activity of Gram-negative and Gram-positive bacteria (Poiată et al. 2009). Antimicrobial activity of Aa is similar to that of the bactericidal antibiotic, streptomycin (Appalasamy et al. 2014). Other candidates for NI include phenolic compounds and other reducing compounds contained in olive mill wastewater (OMW) (Rice and Pancholy 1974; Mishra et al. 1980; Gamba et al. 2005; Mekki et al. 2006).

In this study, we use the vegetable crop, celery (*A. graveolens* L.) to

(1) investigate the effectiveness of three natural materials, OMW, and hydroalcoholic extracts of the leaves (HLE) of Mp and Aa, as potential alternatives to the synthetic DCD to reduce leaching losses of NO_3^-.
(2) compare the effects of the four NI treatments on activity of NH_4^+-oxidizing and NO_2^--oxidizing bacteria in the soil, as indicated by colony-forming units (CFU).
(3) compare the effects of the four NI treatments on N metabolism of *A. graveolens* L., as indicated by
    (a) activity of nitrate reductase (NR) and glutamine synthetase (GS) in leaves and roots.
    (b) concentrations of amino acids and protein in roots and leaves.
(4) determine whether there is a correlation between NR activity in roots and leaves and the concentration of NH_4^+ in the soil.
(5) determine the feasibility of using one or more of the three natural materials to inhibit nitrification.

Materials and Methods

Preparation of HLE of Mp and Aa

The leaves of plants of the Mp and Aa treatments were separately cut in smaller pieces before drying at 60 °C in the hot-air oven for 2 days. The dried material for each plant species was ground into 1 mm particle size using an electric grinder and 100 g of it was mixed with 1000 mL of ethanol. The mixture was shaken for 20 min, and then the mixture was filtered through a paper filter (Whatman No. 1). The filtrates were evaporated to dryness, and then the dried residues were re-dissolved in 10 mL of ethanol at a temperature of 30 °C in order to obtain a 4% extract of each of the two plant species.

Extraction of OMW on Pumice

The OMW was absorbed at room temperature and atmospheric pressure on pumice powder until saturation at a 1/1 solid/volume ratio. Before absorption, the pumice was first ground and sieved at 100 nm. To perform the absorption, 100 mL of OMW was slowly added to 100 g of pumice, allowing a homogeneous infiltration up to the saturation ratio of the pumice/OMW of 1/1 w/w in order to obtain a natural extract containing 1% of total polyphenol content (TPC). The final mixture was washed with distilled water and oven-dried at 70 °C for 36 h.

Experimental Design

Plants of *A. graveolens* L., 30 days after germination, were planted in a soil-m composed of soil, fine sand (0.2–0.5 mm), and peat containing a low level of nutrients with a volumetric ratio 50/25/25 v/v/v in 1-kg polyethylene jars with a volume of 1.3 L, average radius of 6 cm, and height of 11.5 cm with filter paper at the bottom. The plants were placed in a walk-in growth chamber under environmentally controlled conditions (30/26 °C, 70% relative humidity with a 16/8 light regime).

In order to compare effects of each NI treatment in soil with or without plants, the experiment included a control without NI and without plant (WIP) and a control without NI, but containing a celery plant per pot (WI). The four treatments with NI included a celery plant per pot and one of four nitrification inhibitors: DCD, Aa HLE, Mp HLE, or OMW. All pots were fertilized at the time of planting with $(NH_4)_2SO_4$, for an NH_4^+concentration of 200 mmol kg^{-1}. P and K were also added (30 µg g^{-1} soil-m) using $K_2HPO_4{\cdot}3H_2O$ and K_2SO_4.

A four-factor experiment was set up in a randomized complete block design with four replicates (pots) for each of the controls lacking NI, i.e., WIP and WI, and for each of the four NI treatments, i.e., DCD, OMW, Mp HLE, and Aa HLE. Effects of the four NI treatments, i.e., DCD, OMW, Mp HLE, and Aa HLE, were compared among NI treatments and also compared to the WIP or WI controls. For the DCD treatment, DCD at 1% of phenolic and reducing content was dissolved with $(NH_4)_2SO_4$ in distilled water and mixed homogeneously in the soil-m. The same procedure was carried out for the HLE at 4% for both Aa and Mp following the method of Patra et al. (2002). Only for the sample containing OMW with 1% of reducing compounds, chloride was separately mixed with the $(NH_4)_2SO_4$. DCD was used in the incubation experiments as a synthetic NI, applying a prescribed dosage of DCD, equivalent to 1% of the mass of NH_4^+-N added to the soil. All treatments were irrigated with deionized water at short intervals in order to maintain the soil at constant moisture by controlling the weight of the pot after transplanting the seedlings (0 DAP) until the eighth week (56 DAP). The water holding capacity (WHC) of soil-m was measured before starting, and the final moisture of soil in the pots was adjusted to about 80% of its WHC or at about 25 ± 1% of the soil-m weight. For biochemical analysis, plants were sampled at 35 DAP and 56 DAP just before harvest. At each sampling, four pots for each treatment were randomly selected.

To test the effect of each NI on the activity of the nitrifying bacteria, four pots without plants for each NI treatment and for the WIP control were fertilized as described above and incubated at 30/26 °C day/night air temperature and 70% relative humidity. The moisture of the soils was adjusted to 60% of their WHC and kept constant. The activity of nitrifying in the soil-m was determined for soil samples taken on 0, 14, 28, 35, and 56 DAP.

Preparation of Soil to Determine Activity of Nitrifying Bacteria

For each replicate, a 10-g soil sample was used to determine the activity of NH_4^+-oxidizing bacteria (AOB), and another 10-g soil sample was used to determine the activity of NO_2^--oxidizing bacteria (NOB). The samples were suspended in Erlenmeyer flasks with 90 mL of liquid mineral medium and shaken at 120 rpm for 30 min at room temperature in an orbital shaker. After shaking, the flasks were vortexed for 30 s to ensure complete soil homogenization. Two liquid mineral media by Alexander and Clark (1965) were used. The chemical composition (g/L) of the AOB medium was 0.5 $(NH_4)_2SO_4$; 1.0 K_2HPO_4; 0.03 $FeSO_4{\cdot}7H_2O$; 0.3 NaCl; 0.3 $MgSO_4{\cdot}7H_2O$; 7.5 $CaCO_3$, and the chemical composition (g/L) of the NOB medium was 0.006 $NaNO_2$; 1.0 K_2HPO_4; 0.3 NaCl; 0.1 $MgSO_4{\cdot}7H_2O$; 0.03 $FeSO_4{\cdot}7H_2O$; 0.3 $CaCl_2$, and 1.0 $CaCO_3$.

Preparation of Petri Dishes for Bacterial Counts

To cultivate and enumerate the nitrifying bacteria, 1% agar (Oxoid Bacteriological Agar No. 1) was added to the two liquid mineral media. Phenol red, a pH indicator, was added to the enable enumeration of nitrifiers before sterilization. The initial hydrogen ion activity of the media was adjusted to pH 7 with 0.01 M NaOH, and 20-mL portions were distributed in Petri dishes that were sealed and autoclaved at 121 °C for 20 min. After sterilization, 10 µL 1.0 mg mL^{-1} streptomycin was added to prevent growth of Gram-positive bacteria and Gram-negative bacteria other than Gram-negative nitrifying bacteria, and the hydrogen ion activity of the media was increased to pH 8.2 by the addition of sterile NaOH. The concentration of antibiotic used in the present study, 17 nmol of streptomycin in 20 mL of solution, is below the sensitivity threshold of nitrifying bacteria (data not shown).

Total Aerobic Count of Nitrifying Bacteria

Serial tenfold dilutions were made from the homogenized soil into a liquid mineral medium corresponding to each type of bacteria, according to Elbanna et al. (2012), ranging from 10^{-1} to 10^{-5}. Then 0.1 mL of each dilution was used to inoculate, in triplicate, 15 mL of mineral agar medium per Petri dish.

The media were incubated at room temperature until growth was observed in most of the Petri dishes. The dilutions that showed growth of between 25 and 250 colony-forming units (CFU) were selected to quantify the colonies using the method of Rodríguez et al. (2017). CFU g^{-1} soil was calculated, taking into account the titer of the dilution and the amount of inoculum used.

Chemical and Physical Analyses of OMW

Chemical analyses of OMW were performed according to the analytical procedures set out in the revised Italian official methods of soil chemical analysis (Colombo and Miano 2015). OMW was provided by an olive mill located in Isernia (Molise region, Italy) and produced via olive oil centrifugation using a three-phase process. To determine total reducing capacity (TRC), including phenols, 5 mL of OMW were centrifuged for 5 min at 5000*g*. Next, 0.25 mL of supernatant was then added to 0.5 mL ethyl acetate, and the mixture extract was stirred and centrifuged for 5 min at 5000*g*. The extraction procedure was repeated three times with further additions of ethyl acetate (0.5 mL). Finally, the supernatant was dried at room temperature for about 48 h. The solid extract was solubilized using 0.25 mL of a solution containing methanol and water in the ratio 4/1 v/v, and then the solution was mixed by a vortex mixer for 2 min. The extract was placed in 10-mL tubes to which were added 1 mL of distilled H_2O, 0.9 mL of 0.5 M $NaHCO_3$ (pH 8.5), and 1 mL of diluted acetate 1/10 (v/v). The extract was then stirred for 2 min, and after 2 h, TRC was determined via spectrophotometer at $\lambda = 765$ nm, and TRC of the sample was expressed in mg dm^{-3} of gallic acid (Folin method).

Sampling and Determination of Soil NH_4^+ and Leachate NO_3^-

To measure the concentration of NH_4^+ in the soil-m, samples were collected by soil tube ($H = 15$ cm and $\phi = 2$ cm) from the entire profile of the pots. Next, 5 g of the sample, previously dried, was pulverized in an agate mortar. Soil samples (5 g) were extracted with 2 M KCl (10 mL) according to Nelson (1983). The determination of NH_4^+ in the samples was carried out according to a colorimetric method based on the Berthelot reaction (Nelson 1983).

To measure the concentration of NO_3^- in the soil solution of the soil-m, 10 mL samples of leachate were collected from each pot after a weekly irrigation and stored at 4 °C in a refrigerator until analyzed. The NH_4^+ concentration of the soil-m was determined for soil samples taken on 0, 14, 28, 35, and 56 DAP.

Nitrate was determined by reducing NO_3^- to NO_2^- with zinc and adding sulfanilic acid and *N*-(1-naphthyl)ethylenediamine to produce a red complex in an acid condition. The intensity of the red color was analyzed colorimetrically with a Jasco V530 spectrophotometer at a wavelength of 550 nm (Jeffrey et al. 1989). Soil NH_4^+ was determined using a phosphate/tartrate buffer solution adding salicylate/nitroprusside that was acidified with acid hypochlorite. The blue color formed was intensified with sodium nitroprusside and measured colorimetrically at an OD of 650 nm (Baethgen and Alley 1989). All results are expressed on a dry-soil basis.

Calculation of the Mass Balance of N Fertilizer Between Plant and Soil-m

To calculate the mass balance of N between plant and soil, NH_4^+ and NO_3^- were expressed as mmol N per pot. In soil, NH_4^+ and NO_3^- are distributed differently in the liquid and solid phases. Whereas NO_3^- is an anion less retained than NH_4^+ under conditions of leaching, it is assumed that for about 250 mL kg^{-1} soil-m, NH_4^+ is partitioned between the soil solution and negatively charged mineral and organic soil particles. The sum of mmol of NO_3^- contained in all the samples of leachate collected during the experiment (0–56 DAP) was utilized as an index of cumulative leaching loss of NO_3^--N from soil-m. Therefore, the concentration of NH_4^+ in the soil was expressed in mmol NH_4^+ kg^{-1} DW_{soil} and NO_3^- in mmol NO_3^- L^{-1}. Every millimole of NO_3^- in 250 mL of soil solution causes a concentration increase of 4 mM. The mass balance of N was calculated using the algebraic equation between (1) the N used for fertilization and (2) the sum of (a) N assimilated by the plant, (b) the N content of the soil-m, and (c) N collected in the leachate. The N assimilated per plant was calculated as the difference between total N in the roots and shoots of plants at 56 DAP and 0 DAP.

Biochemical Analysis

Sampling of Leaves and Roots

For every pot examined at 35 and 56 DAP, part of the sampled roots and three younger, fully expanded leaves were ground in liquid N_2 to a fine powder and stored at − 40 °C to

be used for biochemical and elemental analyses. At 56 DAP the shoots were harvested. Fresh weight (FW) of shoots and roots was determined before they were treated with liquid N_2, ground to a fine powder, and stored separately at − 40 °C for subsequent chemical analyses.

NR and GS Assays

Aliquots of fine-powdered samples of leaves (300 mg FW) and roots (1 g FW) were treated in 10 mL extraction buffer containing 10% of glycerol (v/v), 0.25% Triton X-100 (w/v), 50 mM Hepes/KOH pH 7.5, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM phenylmethylsulphonylfluoride, (PMSF). Polyvinilpolypirrolidone (0.1% w/v) was added just before extraction to absorb polyphenols and other reducing substances. The homogenate was filtered through four layers of muslin and centrifuged at 20,000*g* for 20 min at 4 °C. The supernatant (crude extract) was used to assay NR and GS activities according to Gibon et al. (2004).

The protein content of the root and leaf tissues was determined according to Lowry et al. (1951), using bovine serum albumin as a standard and expressed in mg/g FW. Enzyme-specific activities were expressed in µmol min^{-1} mg^{-1} of protein.

Primary Amino Acid and Protein Analyses

Aliquots of fine, powdered samples of leaves (100 mg FW) and roots (0.5 g FW) were suspended in 2 mL of ethanol/water (80/20 v/v). After 30 min, the suspension was collected and centrifuged. The supernatant was used to determine amino acid content. The primary amino acids were determined by autosampler-assisted pre-column derivatization by *o*-phthaldialdehyde (OPA), separation by reverse-phase high-performance liquid chromatography (HPLC), and fluorescence detection (excitation at 340 nm and emission at 450 nm) (Di Martino et al. 2003). Proline was determined by HPLC as fluorescent 9-fluorenylmethoxycarbonyl derivative (P-FMOC-carbamate) on sample extracts that were previously derivatized by OPA reagent to remove the primary amino acids and fluorometrically detected using excitation at 266 nm and emission at 305 nm (Di Martino et al. 2006).

Calculation and Statistical Analysis

The percentage of change of various metabolites and enzyme activity of treated (T) over untreated (NT) celery plants was calculated as follows: $[(A^{T} - A^{NT})/A^{NT}] \times 100$, where:A^{T} is the content and/or activitiy measured in treated leaves and roots and A^{NT} represents the same variable(s) determined in these two tissues of the WI treatment.

The data of control plants and plants treated with single inhibitors are presented as means ± SD of four samples ($n = 4$) selected randomly out of 20 pots per treatment. Statistical differences were calculated by using the Tukey's test ($p \leq 0.05$). Analysis of variance (ANOVA) was performed by the software IBM® SPSS® Statistics version 22.0 (Anonymous 2014).

Results

Chemical Properties of OMW

The main chemical properties of OMW used in the present study are reported in Table 1. The pH was 5 and the chemical oxygen demand (COD) was 65 g O_2 dm^{-3}. The biological oxygen demand (BOD) was 37 g O_2 dm^{-3}. Concentrations of the anions Cl^- and SO_4^{2-} were 6 and 0.15 mg dm^{-3}, respectively, while the concentrations of the cations Ca^{2+}, Mg^{2+}, Na^+, and K^+ were 25 mg dm^{-3}, 8.5 mg dm^{-3}, 20 mg dm^{-3}, and 7 mg dm^{-3}, respectively. With respect to inorganic N species, the $NH_4^+$and NO_3^- concentrations were 140 mg dm^{-3} and 3 mg dm^{-3}, respectively. The total phenolic compounds (TPC) concentration was 7 g dm^{-3} , while total suspended solids (TSS) were 85 g dm^{-3}.

Activity of Nitrifying Bacteria in Soil-m

The activity of nitrifying bacteria in soil-m was measured for the control (WIP) and for four NI treatments in pots without plants to test the ability of the nitrification inhibitors to decrease the growth of nitrifying bacteria.

"To determine the activity of AOB or NOB present in soil treated with the NIs, bacteria were cultured in a medium that contained either NH_4^+ OR NO_2^-. Those bacteria that grew in either one of these cultures, as assayed by CFUs, were determined to have nitrifying activity specific to NH_4^+ or NO_2^-.

Table 1 Chemical properties of olive mill wastewater (OMW) sample

Parameter	Value	Unit	Parameter	Value	Unit
pH	5	$-\log[H^+]$	TPC	7	g dm^{-3}
COD	65	g O_2 dm^{-3}	TSS	85	g dm^{-3}
BOD	37	g O_2 dm^{-3}	NH_4^+	140	mg dm^{-3}
Cl^-	6	mg dm^{-3}	NO_3^-	3	mg dm^{-3}
Na^+	20	mg dm^{-3}	K^+	7	mg dm^{-3}
Ca^{2+}	25	mg dm^{-3}	Mg^{2+}	8.5	mg dm^{-3}
SO_4^{2-}	0.15	mg dm^{-3}			

COD chemical oxygen demand, *BOD* biological oxygen demand, *TPC* total phenolic compounds, *TSS* total supspended solids

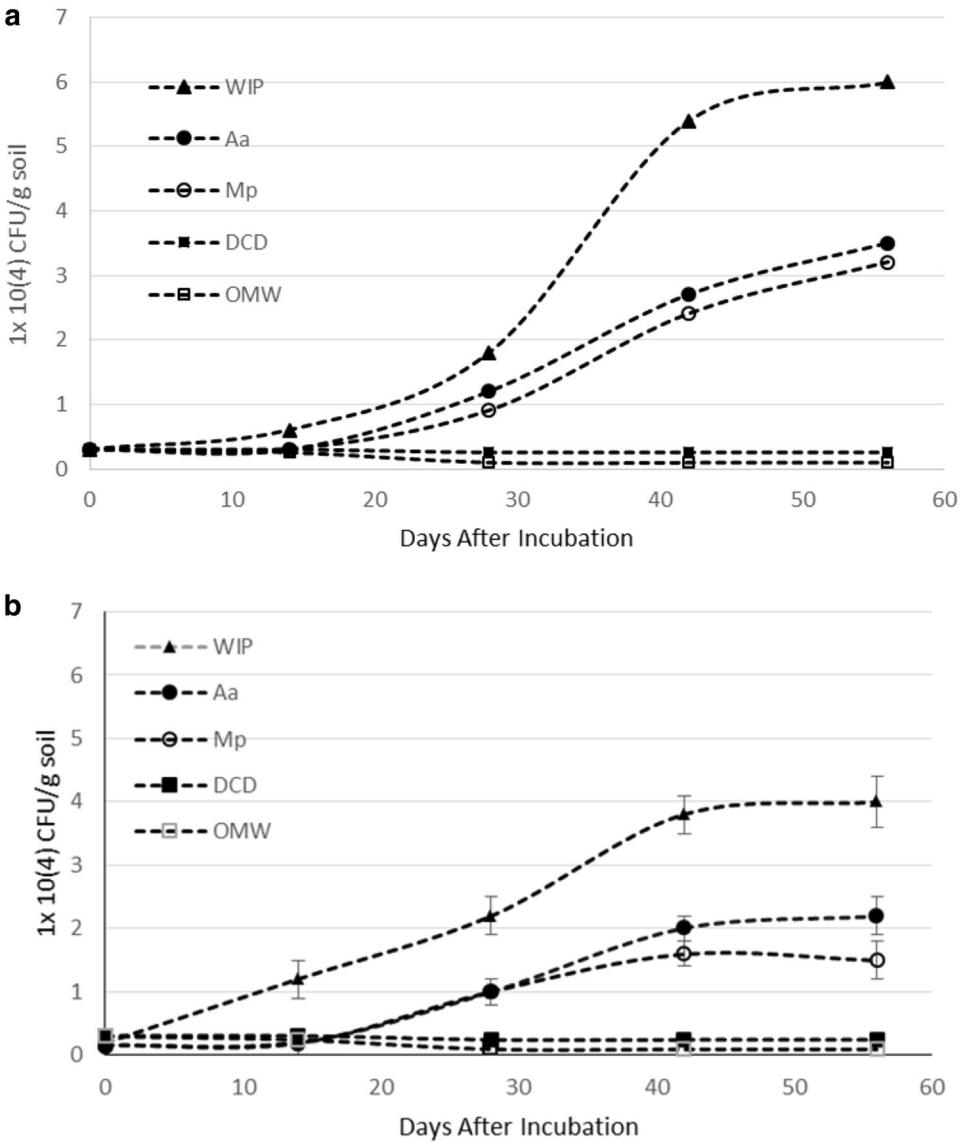

Fig. 1 **a** Activity of NH_4^+-oxidizing (AOB), nitrifying bacteria in the soil-m with three different nitrification inhibitor (NI) treatments as a function of the time. WIP = Control soil with no plant and no NI; OMW = soil + olive wastewater; DCD = soil + dicyandiamide; Aa = soil + *A. annua* L. extract; Mp = soil + *M. piperita* L. extract. CFU = colony-forming units. Bars of means correspond to ± standard error (SE) ($n = 3$). **b** Activity of NO_2^- oxidizing (NOB), nitrifying bacteria in the soil-m with different nitrification inhibitor (NI) treatments as a function of the time. Treatments: WIP = Control soil with no plant and no NI; OMW = soil + olive wastewater; DCD = soil + dicyandiamide; Aa = soil + *A. annua* L. extract; Mp = soil + *M. piperita* L. extract. CFU = colony-forming units. Bars of means correspond to ± standard error (SE) ($n = 3$)

In the presence of NH_4^+ or NO_2^- in soil-m, the number of colony-forming units (CFU) of AOB or NOB treated with Aa HLE and Mp HLE was consistently lower, compared to the control (WIP) (Fig. 1a, b), but sufficiently high enough not to impede the nitrification process. The amounts of viable AOB and NOB were more than 3 and $1.5. \times 10^4$ CFU at 56 at days of incubation, (DAI), respectively, or about half of the respective controls. The more effective nitrification inhibitors, DCD and the natural inhibitor OMW, not only did not allow colony development, but also reduced pre-existing activity of 0.3×10^4 CFU for both AOB and NOB (Fig. 1a, b). Reduced AOB activity is the direct cause of a reduced NOB activity, because the two processes are metabolically linked. This results in the almost total absence of nitrifying bacteria of the DCD and OMW treatments in the testing medium for periods of more than 60 days.

Nitrification Inhibitors' Influence on NO_3^- Leaching and Soil NH_4^+

The NH_4^+ concentration in the soil-m of all treatments decreased from 7 to 56 DAP (Fig. 2). In contrast, the patterns of NO_3^- concentration in the leachate differed among the treatments. In leachate of the control pots, the concentration of NO_3^- increased up to 200 mmol L^{-1} corresponding to 25% of the NH_4^+added during the first 5 weeks. The subsequent decrease to 55 mmol L^{-1} at 56 DAP for the WI treatment completes an approximately bell-shaped curve. During the same period, but with a distinctly linear trend, the concentration of NH_4^+ in the soil-m of the control pots (WI) declined to 30 mmol kg^{-1} $DW_{soil\text{-}m}$ at 56 DAP. The NO_3^- concentration in the leachate of Aa treatment increased from approximately 0 mmol L^{-1} on 0 DAP, increasing to approximately 150 mmol L^{-1} at about 28–35

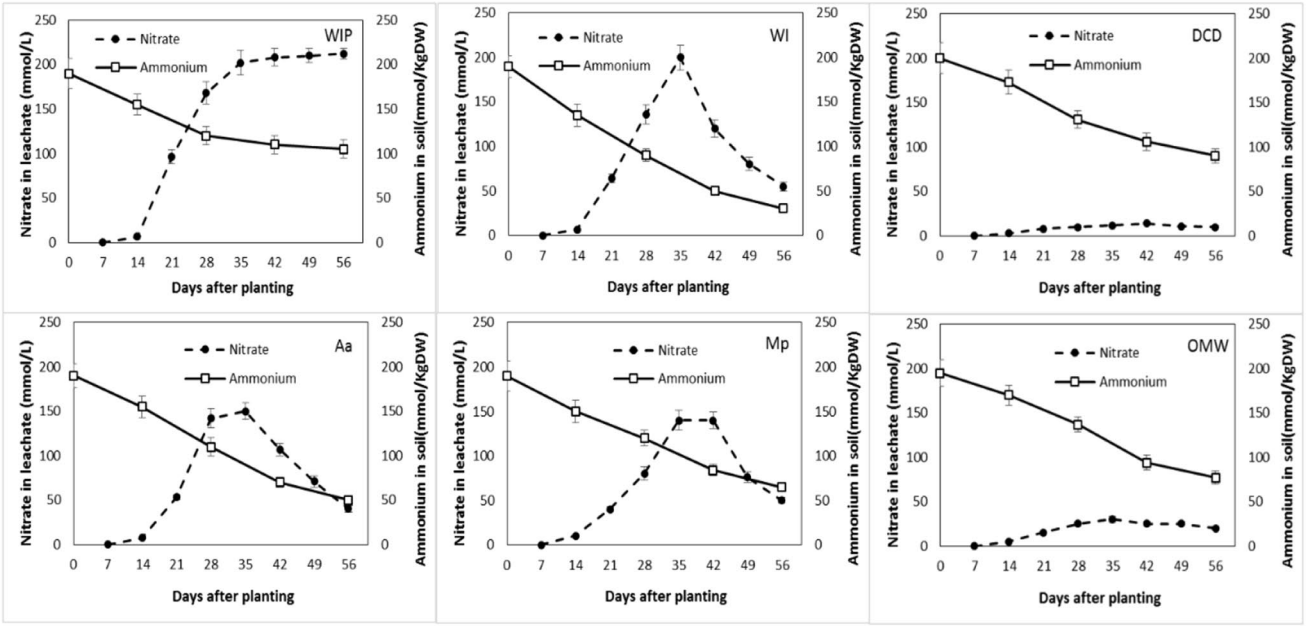

Fig. 2 Mean concentration of NO_3^- in the leachate (dashed line with solid circles) and NH_4^+ in the soil mixture (solid line with open squares) for pots treated with no nitrification inhibitor (NI) or one of four nitrification inhibitors. Treatments: WIP = control with no plant and no NI; WI = control with plant but no NI; DCD = dicyandiamide; Aa = *A. annua* L. extract; Mp = *M. piperita* L. extract; OMW = olive mill wastewater. Bars of means correspond to ± standard error (SE) ($n = 4$)

DAP, and then declined to about 50 mmol L^{-1} at 56 DAP (Fig. 2). The pattern of increase, plateau, and decrease of NO_3^- concentration for the Mp treatment is similar to that of the Aa treatment, but the plateau is approximately a week later, i.e., from 35 to 47 DAP (Fig. 2). The DCD treatment resulted in the NO_3^- concentration in the leachate remaining at approximately 5 mmol L^{-1} or less during the 56-day experimental period. Similarly, the OMW treatments resulted very low concentrations of NO_3^- in the leachate that did not exceed about 20 mmol L^{-1} (Fig. 2).

N Assimilation in the Celery Plant

NR and GS are critical enzymes of N assimilation by plants, and most plant species have the ability to assimilate NO_3^- through reduction to NO_2^- in both roots and shoots and then to NH_4^+ (Epstein and Bloom 2005). The activity and distribution of NR and GS were determined in roots and leaves of control plants and treated plants at 35, and at 56 DAP (Fig. 3). At 35 DAP, low levels of NR activity were detected in the leaves and roots of all plants observed, including the plants grown in the pots of the WI treatment, in which the NO_3^- concentration in the leachate was at the highest value (Fig. 2). On the other hand in the same control pots, where the NH_4^+ concentration in the soil-m was much less than treated pots, significant levels of GS activity were identified in the roots as well in the leaves, about 25 and 30 nmol·min^{-1} mg^{-1}prot, respectively. These values indicated enzymatic catalysis of the glutamine: glutamate coming from metabolism and NH_4^+ absorbed directly by the plants. At 56 DAP, the concentration of NH_4^+ in the soil-m of DCD and OMW treatments was still quite high (90 mmol kg^{-1} DW of soil-m for DCD and 77 mmol kg^{-1} DW of soil-m for OMW). Interestingly this corresponds to a 50% and 35% increase in GS activity in leaves and roots, respectively, when compared to control.

The correlation between enzyme activity and ammonium concentration in the soil mix is shown in Fig. 4 with a significant coefficient of determination (R^2) of 0.90 and 0.75 for roots and leaves, respectively.

Concentrations of Total Protein and Amino Acids in Celery Plants of Different NI Treatments

Concentrations of key nitrogenous compounds of cell metabolism, amino acids, and total protein, were determined at 56 DAP in roots and leaves of celery plants of NI treatments and of the control (Fig. 5). Concentrations of the compounds were higher in celery plants treated with DCD and OMW than in plants of the control treatment WI; no significant differences were evident among Mp, Aa, and WI treatments. Plants growing in soil treated with DCD or OMW, where maximal inhibition of nitrification occurred, exhibited increases of total protein, compared to the control, that were 38.30% and 30.25%, in roots and leaves, respectively (Fig. 5a, b). At 56 DAP, strong changes in

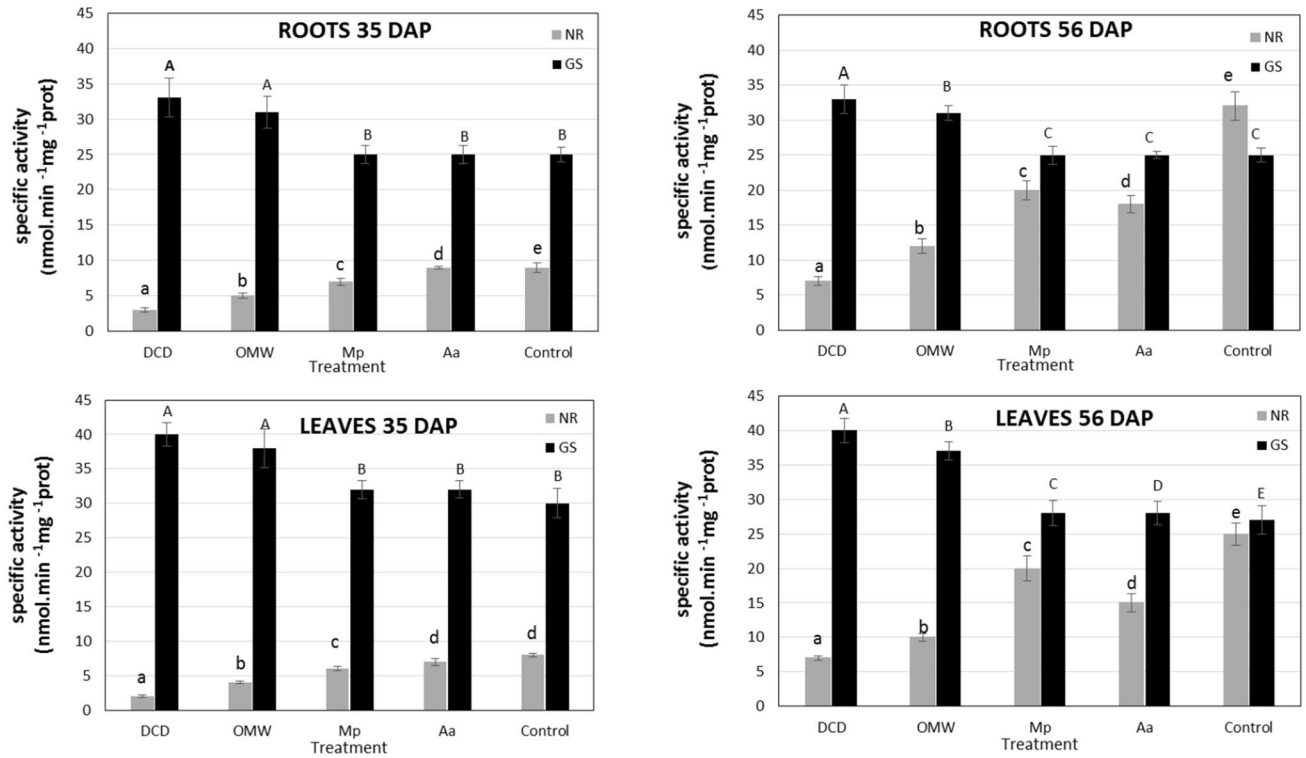

Fig. 3 Activity of nitrate reductase (NR, gray bar) and glutamine synthetase (GS, black bar) in roots and leaves of celery plants sampled at 56 days after planting (DAP). Specific activities are expressed in nmol min^{-1} mg^{-1} protein (prot.). Mean values marked by common letters are not statistically different ($P < 0.05$) according Turkey test performed between treated and untreated samples. Different capital letters indicate statistically significant difference in GS activity; and differences among small letters indicate statistically significant differences in NR activity. Treatments: DCD = dicyandiamide; OMW = olive mill wastewater; Mp = *M. piperita* L. extract; Aa = *A. annua* L. extract; Control = no NI. Bars of means correspond to ± standard error (SE) ($n = 4$)

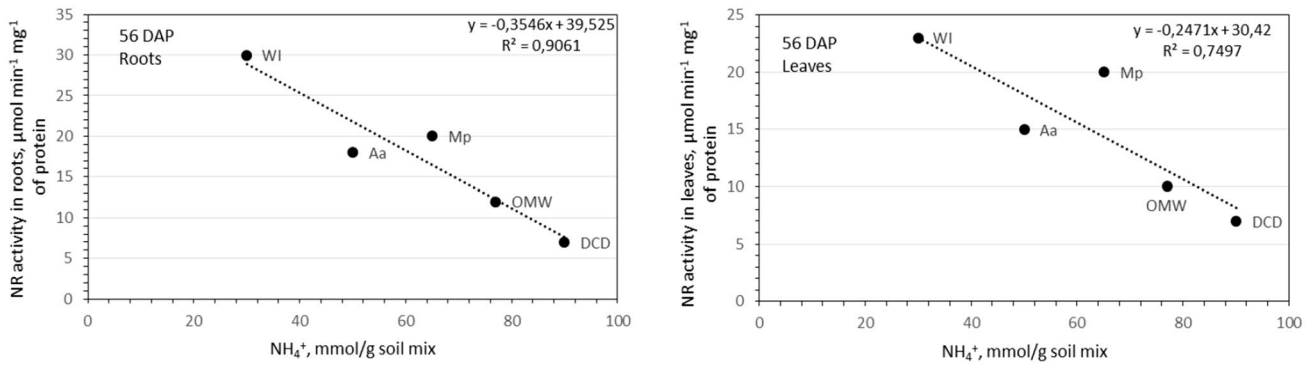

Fig. 4 Relationship between NR activity in the roots (left) and leaves (right) as a function of NH_4^+ concentration in the soil mix. Nitrification inhibitor (NI) treatments: WI = Control, no NI; Aa = *A. annua* L. extract; Mp = *M. piperita* L. extract; OMW = olive mill wastewater; DCD = dicyandiamide. Each data point represents the means of four plant samples

concentration and distribution of free amino acids in roots and leaves also occurred (Fig. 5a, b), compared to the control plants. Total free amino acids increased by about 80% in both roots and leaves of plants growing in soil treated with DCD or OMW, compared to control plants. Among the main amino acids contributing to the overall amino acid concentrations in roots, glutamine, asparagine, arginine, and alanine, but also citrulline and ornithine, displayed the largest increase, reaching concentrations more than 100% higher, while concentrations of glutamate and aspartate showed increases of 60% and 40%, respectively (Fig. 5a). In the leaves of the plants treated with DCD

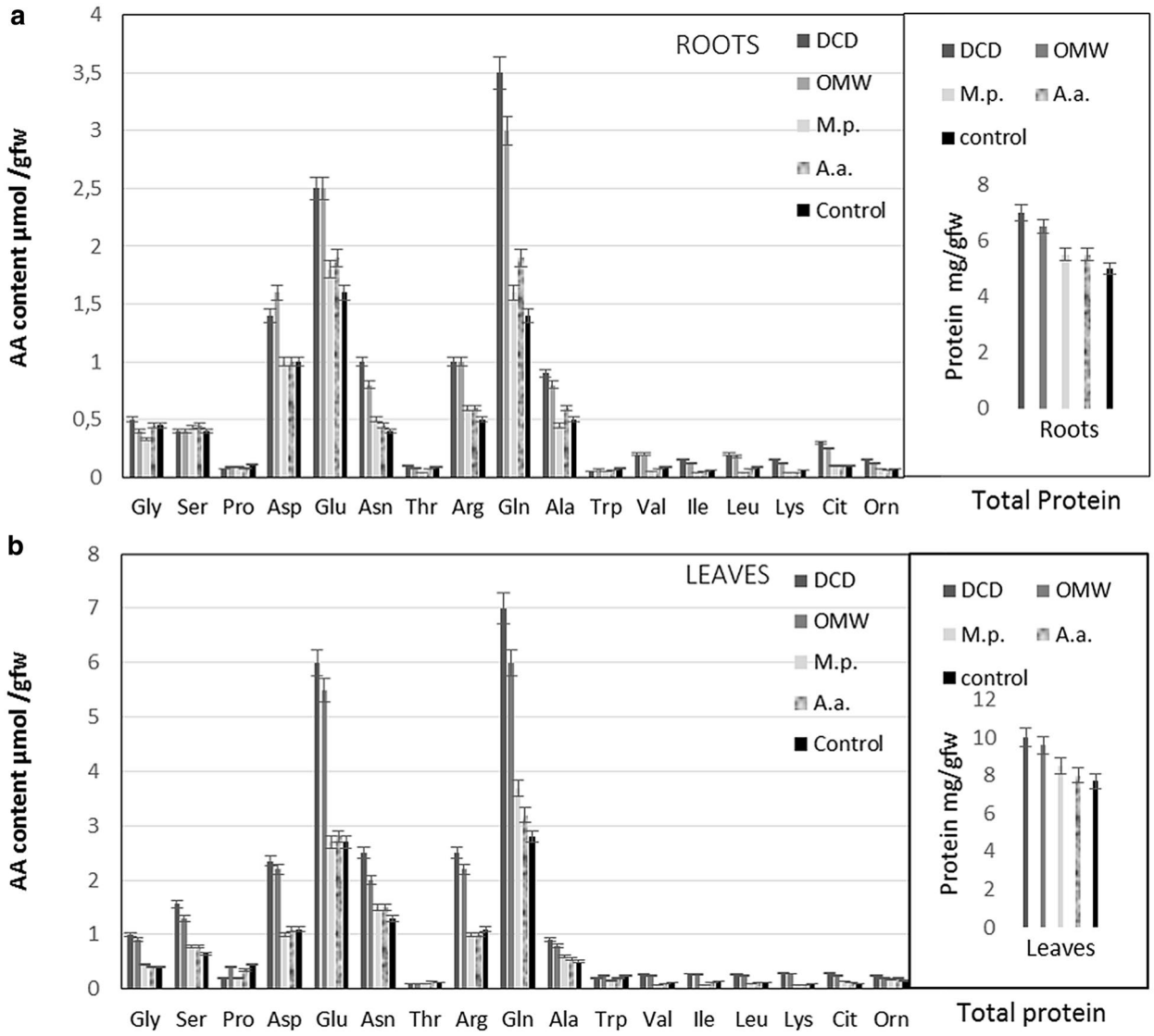

Fig. 5 Free amino acid concentrations and total protein in roots (**a**) and leaves (**b**) by plants collected at 56 DAP after growth with different nitrification inhibitor (NI) and fertilization with 200 mmol NH_4^+ kg^{-1} $DW_{soil\text{-}m}$. Control = no NI; DCD = dicyandiamide; Aa = *A. annua* L. extract; Mp = *M. piperita* L. extract; OMW = olive mill wastewater. The data are presented as mean ± standard error (SE) ($n = 4$)

or OMW, only the glutamine concentration increased more than 150%, glutamate and aspartate concentrations increased about 100%, and asparagine, arginine, and alanine concentrations increased < 100% (Fig. 5b), compared to the control. The pool of other amino acids (valine, leucine, isoleucine, lysine, tryptophan and proline) was approx. 1% of the overall amount in roots as well as in leaves. Only traces of glycine and betaine were found. Among the other amino acids, isoleucine, leucine, valine, and lysine which are among the essential amino acids in animal and human nutrition and citrulline and arginine with a high N/C ratio increased their concentrations more than twofold in the roots and leaves of DCD- and OMW-treated plants, compared to the control.

Fertilizer N: Distribution Between Plant, Soil, and Loss by Way of Leaching

To obtain the mass balance of N fertilizer between plant and soil-m, total N was determined in both plant and soil-m for all treatments except the WIP treatment which contained neither plant nor NI. The dry weight average and N content of six plants was determined at 0 and 56 DAP for each NI treatment (Table 2).

There were no significant differences among treatments for the dry weight of root and shoot and the total N content at both 0 and 56 DAP (Table 2). The NI treatments resulted in differences in the availability of NH_4^+ in the soil-m and oxidation of NH_4^+ to the NO_3^-, as indicated

Table 2 Means of dry weight (DW) and total N (N_{tot}) concentration on a DW basis in root and shoot of six plants at 0 (A) and 56 (B) days after planting (DAP)

O DAP (A) Treatment	Dry weight		Total N	
	Root g DW	Shoot g DW	Root N_{tot} mg/g	Shoot N_{tot} mg/g
WI	4 ± 0.5a	7 ± 1a	15 ± 2a	20 ± 2a
DCD	3 ± 0.4a	8 ± 0.9a	14 ± 1.5a	19 ± 2a
OMW	5 ± 0.5a	7 ± 0.7a	15 ± 1.5a	21 ± 2a
Aa	4 ± 0.4a	9 ± 1a	16 ± 2a	20 ± 2a
Mp	4 ± 0.5a	7 ± 0.8a	15 ± 1a	22 ± 2a
56 DAP (B) Treatment	Dry weight		Total N	
	Root g DW	Shoot g DW	Root N_{tot} mg/g	Shoot N_{tot} mg/g
WI	10 ± 1a	34 ± 3a	22 ± 2a	28 ± 3a
DCD	8 ± 1a	36 ± 4a	21 ± 2a	29 ± 2a
OMW	10 ± 1a	36 ± 3a	22 ± 2a	30 ± 3a
Aa	9 ± 1a	35 ± 3a	20 ± 2a	28 ± 2a
Mp	9 ± 1a	34 ± 4a	21 ± 2a	27 ± 3a

Treatments: WI = Control with plant, but no nitrification inhibitor (NI); DCD = dicyandiamide; OMW = olive mill wastewater; Aa = *A. annua* L. extract; Mp = *M. piperita* L. extract

In each column, values marked by common letters are not statistically different at $P \leq 0.05$ according to Tukey's test performed between the different NI treatments. The WI control and the four NI treatments, DCD, OMW, Aa, and Mp, are contained one plant per pot, and each value is the mean ± SD ($n = 6$)

Table 3 Mean value of N assimilated per plant (N_{ass}), total N (N_{tot}) in 1 kg soil-m, and N leached ($N_{leached}$) of six samples (pots) at 56 DAP

Treatment	N_{ass} per plant		N_{tot} per pot (1 kg)		$N_{leached}$
	0 DAP	56 DAP	0 DAP	56 DAP	56 DAP
	mmol N/plant		mmol N/pot		mmol N/plant
WI	0	69 ± 7a	190 ± 2a	45 ± 4a	76 ± 6a
DCD	0	72 ± 6a	190 ± 2a	100 ± 8b	18 ± 2b
OMW	0	77 ± 6a	190 ± 2a	93 ± 10b	20 ± 2b
Aa	0	65 ± 6a	190 ± 2a	65 ± 5c	60 ± 5c
Mp	0	64 ± 5a	190 ± 2a	75 ± 7c	51 ± 5c

Treatments: WI = Control with plant, but no nitrification inhibitor (NI); DCD = dicyandiamide; OMW = olive mill wastewater; Aa = *A. annua* L. extract; Mp = *M. piperita* L. extract

Each value is the mean ± SD of six replicates. In each column, values marked by common letters are not statistically different at $P \leq 0.05$ according to Tukey's test performed between the different NI treatments

by the different concentrations of NH_4^+ in the soil-m and NO_3^- in the leachate (Fig. 2). At 56 DAP there were no significant differences among treatments in the values of N assimilated (N_{ass}) per plant, but there were significant differences among treatments for the number of moles of total N (N_{tot}) remaining in the soil-m per pot and the number of moles of N lost due to leaching ($N_{leached}$) per pot (Table 3).

Discussion

Inhibition of Activity of Nitrifying Bacteria

DCD is one of the most efficient and used chemical nitrification inhibitors in agriculture. According to Amberger (1989), DCD inhibits oxidation of NH_4^+ by deactivating ammonia monooxygenase (AMO) enzyme of the AOB. The deactivation of the AMO enzyme decreases the growth rate of *Nitrosomonas* bacteria and, consequently, the metabolic conversion rate of NH_4^+ to NO_2^-. Plants can also decrease the activity of nitrifying bacteria in the soil by the action of tannins that are naturally produced in the plant. One of the main characteristics of tannins is the formation of insoluble complexes with proteins causing the proteins to precipitate (Pietta et al. 2003). Most dicotyledonous species are tannin free (tested by their ability to precipitate proteins) (Mole 1993). The toxicity of tannin is quite broad spectrum, affecting many microorganisms, as well as AOB, and the antioxidant and bactericidal activity of some leaf extracts such as *Mentha piperita* and *Artemisia annua* against some Gram-positive and Gram-negative bacteria are widely reported in the literature (Singh et al 2011; Martini et al. 2020).

Three plant extracts, OMW, Aa, and Mp, and the synthetic compound DCD lowered the activity of nitrifying bacteria in soil, compared to the control. Treatments Aa and Mp were effective in decreasing the growth of AOB

during the 56 days that the bacteria in the presence of added NH_4^+ (200 mmol kg^{-1}) (Fig. 1a), indicating that these two hydroalcoholic extracts were capable of inhibiting the first step of nitrification, i.e., oxidation of NH_4^+ to NO_2^-. The inhibition of NOB by treatment with Mp and Aa (Fig. 1b) could result from a lack of nitrous substrate The first and second step of nitrification were inhibited by the synthetic NI, DCD, and by the OMW, because they were capable of breaking down the pre-existing latent population of both AOB and NOB. (Fig. 1a, b). Treatment with Aa or Mp was 45 and 55% less effective in reducing the number of nitrifying bacteria when compared to the control. Figure 1a, b show the growth curves of nitrifying bacteria from the latent state before the ammonium addition at day 0. In the presence of added NH_4^+ (200 mmol kg^{-1}), the activity of AOB of the control without NI (WIP) (Fig. 1a) increased until it reached values of 6×10^4 CFU at 56 DAP, approximately 200 times the initial activity of the AOB at 0 DAI. The inhibition of growth of AOB and NOB by DCD and OMW was extreme and was more effective than inhibition by the plant extracts of the Aa and Mp treatments. The curves showing CFU of AOB (Fig. 1a) and NOB (Fig. 1b) for the four NI treatments indicate that OMW and DCD were the most effective inhibitors that prevented growth of AOB and NOB, diminishing the risk of producing excessive amounts of nitrate in the soil that could be leached into aquifers.

Nitrification Process Inhibition and NO_3^- Leaching Reduction

Compared to the WIP and WI treatments, the ability of the four nitrification inhibitors (DCD, Aa, Mp and OMW) to decrease nitrification resulted in less NO_3^- lost in the leachate (Table 3). In fact, the highest concentration of NO_3^- in the leachate of the DCD treatment was only about 14 mmol L^{-1}, corresponding to 5% of NH_4^+ added (Fig. 2). Among the three natural inhibitors, OMW displayed effects on concentrations of NH_4^+ in the soil-m and NO_3^- in the leachate that are most similar to those of DCD (Fig. 2). Comparing concentrations of NO_3^- in the leachate of both OMW and DCD to the WIP treatment, the two nitrification inhibitors reduced to about 10% the oxidation of the NH_4^+ added, while maintaining its concentration an adequate level for plant nutrition, as indicated by no significant differences in DW of root and shoot for untreated control (WI) and the four NI treatments at 56 DAP (Table 2). At 35 DAP, the NO_3^- concentrations in the leachate of pots treated with Mp and Aa corresponded to 15 and 20% of the NH_4^+ added to soil-m (Fig. 2). At 56 DAP, as a result of nitrification inhibition, the cumulative leaching losses of soil NO_3^--N were 24%, 26%; 67%, and 79% of the control for the DCD, OMW, and treatments, respectively (Table 3). One of the aims of this study was to investigate the ability to retard the nitrification process using natural materials such as OMW, Mp, and Aa. The measured inhibitory effects of DCD and OMW (Fig. 1b) on oxidation of NO_2^- to NO_3^- resulted in a continuous NH_4^+ persistence in the soil-m solution during over 56 days of the experiment (Fig. 2). In addition, inhibitory effects of DCD and OMW on the growth of NO_2^--oxiding bacteria were comparable (Fig. 1b) and resulted in significantly slow NO_3^- release, during 56 days of the experiment (Fig. 2). These results are analogous and support the research of Di and Cameron (2002), who found that NH_4^+ from $(NH_4)_2SO_4$ was present in soil for up to 60 days when DCD was used as a synthetic N inhibitor.

In the pots treated with DCD or OMW, there were significantly greater levels of NH_4^+ in the soil-m, compared to the control (WI), Aa and Mp treatments at 56 DAP (Table 3). The NO_3^- concentration in the leachate reached after 28 DAP was relatively low and varied little through the period ending at 56 DAP (Fig. 2). On the other hand, the concentration of NO_3^- in the leachate of pots treated with Mp reached a maximum, approximately 120 mmol L^{-1}, in the range of 35–42 DAP before declining. Showing the same general trend, the NO_3^- concentration in the leachate of pots treated with Aa increased from 7 to 42 DAP, reaching a maximum of about 150 mmol L^{-1}, before declining. The N level, (NH_4^+ plus NO_3^-) of the control (WI) and the four NI treatments (Fig. 2) was sufficient to satisfy the needs of the celery, regardless of leaching losses of NO_3^-, during the 56-day experimental period, as indicated by no significant ($P < 0.05$) difference in root or shoot DW among all treatments (Table 2).

In the control (WI) pots, NO_3^- leaching began at 14 DAP and then increased to the maximal value at 35 DAP and then declined with a concurrent substantial reduction of the NH_4^+ concentration in the soil-m. Treatments with DCD and OMW, as expected, maintained low NO_3^- and elevated NH_4^+ soil-m levels during the experiment. Thus, the inhibitory effects of DCD and OMW on the nitrification process maintained a relatively high concentration of NH_4^+ in the soil-m ensured efficient N assimilation in the plant during the 56-day experimental period, enabling better nutritional status and N metabolism by an active synthesis of organic N compounds (Masclaux-Daubresse et al. 2006). A balance of the N mass between plant and soil-m, compared to the added N, gives an idea of the amount of N removed by leaching. In The data of Table 3 show that after fertilizing with 200 mmol NH_4^+-N, the cumulative N leached, as a percentage of the 200 mmol N added to the soil-m by fertilization was 38%, 30%, 26%, 10%, and 9%, respectively, for WI; Aa; Mp; OMW, and DCD treatments.

Influence of NI on Nitrification During N Assimilation by Celery

Figure 2 shows three patterns of mean NO_3^- concentration of the leachate for the six treatments. The first pattern is represented by control treatment, WIP, shows the effects of soil microbes on nitrification without a plant. The WIP treatment resulted in a gradual decrease in NH_4^+ in the soil-m from 14 to 56 DAP and increasing presence of NO_3^- in the leachate from 7 DAP, reaching an approximately steady state from 35 to 56 DAP. The constant concentration of NO_3^- in the leachate of the WIP control from 35 to 56 may indicate a feedback mechanism by which high NO_3^- concentration in the soil solution inhibited further increases in nitrification beginning at 35 DAP, or a sink for NO_3^-, such as denitrification, may have been present in the soil-m, resulting in an equilibrium concentration of 200 mmol NO_3^-/kg DW. If a portion of the soil-m of the WIP treatment had abundant soil water and sufficient substrate to supply energy, denitrifying bacteria could convert NO_3^- into volatile forms with gaseous loss into the atmosphere (Rendig and Taylor 1989).

The second pattern is represented by three treatments, i.e., the WI control without NI, and the Mp and Aa treatments, which showed a moderate capability to inhibit nitrification in the presence of a celery plant (Fig. 2). During the period of 0–56 DAP, these treatments displayed decreasing NH_4^+ concentration in the soil-m. Of these three treatments, the greatest concentration of NO_3^- measured in the leachate at 35 DAP was with WI treatment. At 35 DAP, the concentration of NO_3^- in the leachate began to decline precipitously after 35 DAP, suggesting that absorption of NO_3^- through the roots of the celery plant removed NO_3^- from the soil solution, resulting in a lesser concentration of NO_3^- in the leachate through day 56. For the WI control, 35 DAP was the tipping point, when the absorption of the plant began to exceed the rate of production of NO_3^- by microbial nitrification. As the celery grew from 35 to 56 DAP, it acted as a progressively stronger sink for NO_3 that nitrifying bacteria were producing from NH_4^+ in the soil-m. The Aa and Mp treatments resulted in lower values of NO_3^- concentration in the leachate at their maximal values (approximately 150 mmol L^{-1}) between 28 and 35 DAP, compared to the maxima of NO_3^- concentration of the WIP and WI treatments (200 mmol L^{-1}), indicating nitrification inhibition of these two plant extracts was occurring. During the period of 35 DAP to 56 DAP, the plants of the WIP control and of the Aa and Mp treatments were sinks for both NO_3^- and NH_4^+ from the soil solution, and the NH_4^+ in the soil solution was being consumed by both nitrifying microbes and the plants. In the assays of the activity of nitrifying bacteria, the Aa and Mp treatments clearly decreased the activity of both NH_4^+-oxidizing (Fig. 1a) and NO_2^--oxidizing bacteria (Fig. 1b), compared to the WIP control without NI and without plant, explaining why in the 56-day experiment with one celery plant per pot, NO_3^- concentrations in the leachate of the Aa and Mp treatments were lower, compared to the WI control.

The third pattern of NO_3^- concentration in the leachate is represented by the DCD and OMW treatments that proved to be relatively strong nitrification inhibitors, compared to the Aa and Mp treatments (Fig. 2). Like the other four treatments, the DCD and OMW treatments displayed decreasing NH_4^+ concentration in the soil-m during the 56-day experiment. However, both the DCD and OMW treatments dramatically decreased the concentration of NO_3^- in the leachate, indicating that both the synthetic product DCD and the waste by-product of olive production, OMW, were similarly effective in reducing the loss of N by leaching from the pots. The assay of the activity of NO_2^--oxidizing, nitrifying bacteria shows that the DCD and OMW treatments almost completely inhibited oxidation of NO_2^- to NO_3^-, compared to intermediate inhibition of NO_2^- oxidation to NO_3^- by the Aa and Mp treatments, assuming no inhibition of NO_2^- oxidation to NO_3^- in the control (WIP) treatment. Denitrification, the production of N_2O or N_2, that was not measured in our study, may account for losses of another portion of NO_3^--N that was produced by oxidation of the NH_4-fertilizer and subsequent oxidation of NO_2^- during the 56-day experimental period.

Considering that (1) NH_4^+ was transferred from the soil solution through the roots to the rest of the plant, (2) amino acids or other derivatives of NH_4^+ assimilation may activate a transduction signal that represses NR activity as its concentration increases, (Fig. 4) (Gilbert et al 2016) and (3) amino acids or other derivatives of NH_4^+ assimilation may exert an effective inhibition on the net uptake of the NO_3^- (Hachiya and Sakakibara 2017), it is possible that changes in NO_3^- concentrations in the leachate in the first 3 weeks were due to the activity of nitrifying bacteria. Later (56 DAP), when NH_4^+ was still at lower concentrations with significant NO_3^- leachate, WI celery plants showed the highest NR activity compared to the other treatments. (Figs. 3, 4). NR activity may account for the assimilation of the portion of NO_3^- absorbed from the soil solution by the celery plants over the course of the experiment and is probably associated with a decrease of NO_3^- concentration in the leachate due to absorption of NO_3^- and assimilation of N from NO_3^- by reduction and other metabolic reactions in cells of the celery plants of this study.

In plants, NR is a key enzyme in the regulating process of NO_3^- reduction, which in addition to being substrate-induced, could also be repressed by-products such as ammonium or its derivative (Fig. 4). As noted above, NR activities detected were greater in the roots than in the leaves of the WI control plants at 35 and 56 DAP (Fig. 3), whereas the greater GS activity was detected in the leaves, rather than the

roots of plants treated with DCD or OMW. In this context, it was found that the high level and permanence of NO_3^- in soil-m without nitrification inhibitors, may lead to NR activity increase by substrate induction, since NR in plants is an adaptive enzyme which is formed only in the presence of its substrate (Kessler and Oesterheld 1970). On the contrary, the high NH_4^+ and glutamine concentrationjs in roots and leaves resulting from GS activity (Fig. 3), for celery plants grown in the soil-m treated with DCD and OMW inhibitors, suggest a repression of NR enzymatic activity, as reported for other plant species (McCarty and Bremner 1992; Migge et al. 2000; Fan et al. 2006). In this context, the higher levels of NR in leaves and roots of plants treated with Mp and Aa, compared with the plants treated with OMW (Fig. 4), correspond to a higher concentration of NO_3^- leachate (Fig. 2) and to a relatively low inhibitory effect on nitrification, compared to the OMW treatment (Fig. 2). Moreover, the relatively high concentrations of NO_3^- in the leachate of sample WIP, WI, Aa, and Mp plants (Fig. 2) coincide with a lowering availability of NH_4^+ in the soil-m and a reduction of GS activity with respect to DCD and OMW treatments. Although N metabolism in the whole plants, measured by activities of NR and GS activity (Fig. 3), amino acid concentration and total protein (Fig. 5) differed as a function of differing nitrification inhibition in this study, the mean DW and mean N_{Tot} of leaves and roots did not differ among differing treatments. This lack of difference of plant dry weights among treatments indicates that the NI treatments had no detrimental effect on carbon assimilation or anabolic metabolism reflected in the quantity of dry weight produced by the celery plants during the 56-day experimental period.

Nitrogen metabolism is not only one of the primary processes of plants, and it includes biosynthesis of N compounds in plant cells. The assimilation of N by plants is includes the synthesis and conversion of amino acids after assimilation of NH_4^+ absorbed by plant roots from the rhizosphere. Metabolism of N by the plant and includes the reduction of NO_3^--N once NO_3^- has been absorbed by the plant from the rhizosphere. Therefore, patterns of concentration of amino acids in the roots and leaves at 56 DAP provide information on both N metabolism and the physiologic state of the plant, as affected by different natural inhibitor treatments.

Nitrogen Source Discrimination by Celery Roots and Aminoacid Biosynthesis

At 35 DAP, even though the NH_4^+ concentration was lower in the soil of the control (WI) than in the treated samples (Fig. 2), significant levels of GS activity was identified in the roots and leaves of control plants, about 25 and 30 nmol min^{-1} mg^{-1} prot, respectively (Fig. 3). These values indicate enzymatic synthesis of glutamine by the condensation of glutamate from metabolism and NH_4^+ absorbed directly by the plants. Ammonium derived from the reduction of NO_3^- in the plants could be metabolically negligible in the control plants, probably due to low levels of intracellular NO_3^-. In fact, there were no significant differences in NO_3^- concentration between WI and WIP in the soil solutions at 35 DAP. This supports the hypotheses that there is either a lack of NO_3^- uptake at these external NH_4^+ concentrations (Kronzucker et al. 1999) or that internal glutamate and glutamine levels are responsible for downregulating NO_3^- transporter expression (Vidmar et al. 2000). On the other hand, significant differences were not shown between WI and WIP for NO_3^- concentrations in the soil solutions at 35 DAP. However, when the NH_4^+ concentration of the soil-m of control and Mp- and Aa-treated plants dropped to low levels (20, 50 and 45 mmol kg^{-1} $DW_{soil\text{-}m}$) and in presence of NO_3^- concentrations of 45, 75, and 50 mmol L^{-1}, respectively, significant NR activity was detected, indicating that NO_3^- as substrate represented an alternative to NH_4^+ as an N source for the plant. At 56 DAP, i.e., when the NH_4^+ concentration had significantly decreased in the soil-m of all treatments (Fig. 2), the levels of glutamate and glutamine were always the highest, both in the control and treated plants (Fig. 5). In particular, at 56 DAP in DCD- and OMW-treated plants, the concentrations of glutamine (GLN) and glutamate (GLU) in the leaves reached 100% more than in the leaves of the control, whereas in the roots of the DCD- and OMW-treated plants, GLN and GLU concentrations were 100% and 60%, respectively, compared to the roots of the control. GLN and GLU, being, respectively, key sources of nitrogen and precursors for the synthesis of other amino acids, are regulated by a dynamic duo of enzymes, GS and glutamine oxoglutarate aminotransferase (also known as Glutamate synthase, or GOGAT). GLN and GLU are metabolic precursors that contribute to increasing the entire amino acid pool and, particularly, amino acids of the glutamate family, such as alanine, aspartic asparagine, and arginine. Increases of total protein in roots and leaves of plants treated with DCD or OWM, were 38.30% and 30.25% greater, respectively, compared to the control (Fig. 5). Finally, considering the increase of protein and free amino acid concentrations (Fig. 5), among which isoleucine, leucine, valine, and lysine are essential amino acids in animal and human nutrition, it could be suggested that the OMW plant tissues had a higher nutritional value than those of the control.

The most important aspect of the pattern of amino acids concentrations of all plants examined at 56 DAP is that the sum of the concentrations of amino acids is greater in the roots and leaves of plants growing in soil treated with NIs, compared to the roots and leaves of the WI control. Compared to the control, there was an increase of about 80% of the sum of concentrations of all amino acids in both roots and leaves of plants grown in soil treated with DCD

or OMW. The most abundant free amino acids in the roots were glutamine, glutamic acid, aspartic acid, asparagine, and arginine all of which have a high N/C ratio and are involved in N transport and storage in plants. Similarly, in the leaves, the most abundant free amino acids were glutamine, glutamic acid, asparagine, aspartic acid, and arginine. In the leaves, the concentrations of these five amino acids were approximately twice their concentrations in the roots indicating that, in the leaves, the GS-GOGAT cycle was much more active where carbon skeletons and energy supplied by photosynthesis was largely available, compared to the roots where mineral nutrients are absorbed.

General Inhibitory Effects

The results obtained in this research are consistent with those of a similar study that found the application of DCD in soil reduced NO_3^- leaching by 22% (Kim et al. 2014). Other studis showed that use of a NI reduced leaching losses of NO_3^- by about 50–60% (Di and Cameron 2005, 2006; Singh and Verma 2007). Our results indicate that OMW is less expensive and locally available in the Mediterranean region and is a potent NI, compared to DCD which is expensive and difficult to apply in the field. The similar, inhibitory effect of OMW (56%) at day 28 relative to 1% dose of DCD (66%) seems to indicate that OMW may be a valid alternative to the synthetic NI DCD. Our findings are different from those of Kiran and Patra (2003) who found that treating urea with *Artemisia* oil led to a higher recovery of the added N (63%) than did the use of DCD (46%). The differences in the efficacy of the Mp and Aa extract may be due to heterogeneity in their tetranortriterpenoids concentrations. Even though the Mp and Aa extracts were good nitrification inhibitors, their mode of action and the active compound for the inhibition were less effective than were those of OMW. Abbasi et al. (2011) attributed the nitrification inhibition effect to azadirachtin (tetranortriterpenoids) partly because of its strong insecticidal effect (Abbasi et al. 2011). Our results clearly indicate that tannins and polyphenols of OMW, even in solid state, were more active for the inhibition of nitrification of NH_4^+ from an NH_4^+ fertilizer.

Conclusion

The OMW and, to a lesser extent, the less effective extracts of Mp and Aa, are potential alternatives to DCD as a NI. The results obtained by the present study demonstrate that soil treated with OMW or DCD can retain N from an NH_4^+ fertilizer source in the soil-m during the growth vegetative phase of celery, thereby enhancing N nutrition. With less NO_3^- leaching resulting from the use of nitrification inhibitors, the fertilizer N from $(NH_4)_2SO_4$ was used more efficiently, compared to a control treatment to which no NI was added. The results of the present study show DCD to be an efficient NI, and OMW is an almost equally effective, natural alternative. The natural N inhibitors tested in the present study are relatively biodegradable and their ability to reduce leaching losses of NO_3^- when thoroughly blended in a soil mixture suggests an hypothesis to test by further research, i.e., that OMW, Ap, and Mp can reduce leaching losses of NO_3^- when used to coat seeds or fertilizer granules.

Author Contributions CDM, GP, and TWC designed experiments and supervised the study. EDI and CDM performed sample preparation and conducted the experiment. CDM and GP performed the biochemical analyses. All authors contributed to the analysis and interpretation of data. TWC contributed to the final version of the manuscript. All authors provided critical feedback and helped in sample analysis and manuscript writing.

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