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Full Length Research Paper

Phenolic acids and total antioxidant activity in *Ocimum basilicum* L. grown under Na_2SO_4 medium

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The antioxidant activity of two basil cvs (*Ocimum basilicum* L. cvs. Genovese and Fine), grown for 15 and 30 days in the absence or in the presence of 25 mM sodium sulphate (Na_2SO_4), was measured. At the same time, phenolic acid contents of the same plant materials were determined to evaluate their probable contribution to the total antioxidant capacity. The results showed that Genovese cultivar was a better source of antioxidant compounds than Fine one, irrespective of the salt and the period of treatment, even if Na_2SO_4 salinity was less stressful for Fine cultivar that after 15 days of treatment was able to increase its antioxidant power in comparison to Genovese one. In addition, although major phenolic acids in Genovese and Fine basil remained constant or decreased with salinity, we observed an increase in hydrophilic antioxidant power either after 15 or 30 days of treatment. The lack of correlation between phenolic acids present in basil and antioxidant activity could be explained by the fact that other antioxidant hydrophilic molecules are synthesised under stress conditions.

Key words: *Ocimum basilicum*, phenolic acids, Na_2SO_4 salinity, hydrophilic antioxidant activity, lipophilic antioxidant activity, $ABTS^+$.

INTRODUCTION

The use of plants as source of antioxidants to enhance health and food preservation is of current interest (Rice-Evans et al., 1997). Epidemiological studies have suggested positive associations between the assumption of diets rich in fruits and vegetables and the prevention of diseases (Scalbert and Williamson, 2000). These health promotion effects have been related to the presence in foods of antioxidant-active components (Kaur and Kapoor, 2001). Antioxidants, synthetic or natural, can be effective in the prevention of free radical formation by scavenging or promoting their decomposition with the consequent suppression of some disorders (Halliwell, 2000). However, there are concerns about the use of synthetic antioxidants, because of their instability and their possible activity as promoters of carcinogenesis (Namiki, 1990). Consequently, during recent years, there is a growing interest in the studies of natural healthy substances and additives (Tomaino, et al., 2005) as potential antioxidants. Several methods have been developed for measuring total antioxidant activity (TAA) of fruit, vegetables, food and beverages because of the difficulty in detecting separately the activity of each antioxidant component (Wang et al., 1997; Benzie and Strain, 1999). Moreover, the sum of their activities might not reflect total antioxidant power since synergism or antagonism among different antioxidants can interfere. For these reasons, the concept of total antioxidant activity was introduced (Pellegrini et al., 1999). TAA takes into account the antioxidant activity of single compounds present in food or biological samples as well as their potential synergistic and redox interactions. A simple scavenging assay, such as the TEAC (Trolox equivalent antioxidant capacity), has been widely accepted as a standard tool to measure the antioxidant activity in the nutraceutical, pharmaceutical, and food industries. In

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general, components in plants can be divided into two fractions, lipophilic and hydrophilic. Although there is no definite clear demarcation between them, the physio-chemical properties of these two groups of components are quite different. Some investigators have proposed that, in order to obtain a good measurement of TAA, lipophilic components need to be separated from those hydrophilic using similar chemical principles (Arnao et al., 2001).

Many herb spices, especially those belonging to the Lamiaceae family show strong antioxidant activity (Hirasa and Takemasa, 1998). *Ocimum basilicum* L., a member of the Lamiaceae family, is known as aromatic and medicinal plant and is widely cultivated in many countries. In view of its several therapeutic potential and its importance as a basic component of the Mediterranean diet, basil deserves scientific attention. *O. basilicum* contains vitamins and phenolic compounds (Tarchoune et al., 2009, 2010, 2012; Sgherri et al., 2010) acting as powerful antioxidants and free radical-scavengers. Their concentrations are influenced by many environmental factors such as salinity (Shannon et al., 1994), which affects more and more seriously agricultural yields in the countries of the Mediterranean basin. In particular, the presence of saline soils and the use of groundwater for growing plants are more and more widespread, especially in arid and semi-arid regions. Saline soils in nature are normally a mixture of different salt species among which sulphate and chloride salts often dominate. Whereas a lot of studies have been done on NaCl, little attention has been given so far to other salts such as Na_2SO_4 (Tarchoune et al., 2010, 2012).

The object of the present work was to study the effects of 25 mM Na_2SO_4 on two cvs. (Genovese and Fine) of *O. basilicum* L. grown for 45 and 60 days. In particular, antioxidant activity of lipophilic and hydrophilic extracts from leaves, and phenolic acid composition were analysed.

MATERIALS AND METHODS

Chemicals

All reagents were of the highest purity and were purchased from Sigma-Aldrich (Milan, Italy). Water was of Milli Q grade. All solvents and water were accurately degassed before use in the analyses.

Plant material and culture conditions

Seeds of two commercial cultivars (*O. basilicum* L. cvs. Genovese and Fine) were sown in Petri dishes at 25°C in the dark. After 5 days, uniform seedlings were transplanted in hydroponic medium in pots containing 1 L of diluted (1:8, v/v) aerated Hoagland's solution (Hoagland and Arnon, 1950), with one plant by pot. Solutions renewed every week. Plants were maintained in a growth chamber at temperature of 22/18°C (day/night), 60 to 80% RH, 150 µmol m^{-2} s^{-1} (PAR) with a 16 h photoperiod. Thirty days after germination, plants were divided into two groups. For one group, Hoagland's solution contained 0 mM Na_2SO_4 and it was taken as the control; for the other one (treatment) Hoagland's solution was enriched with 25 mM Na_2SO_4. Leaves were harvested after 15 and 30 days of salt treatment (45-and 60-day-old-plants, respectively). At each harvest, fresh leaves were placed immediately in liquid nitrogen and stored at -80°C till extraction. Some leaves were taken for fresh weight (FW) and dry weight (DW) measurements.

Antioxidant activity (TAA)

Leaves were pulverized with liquid nitrogen using a mortar. The aqueous extract was obtained using Milli Q water accurately degassed containing 5 mM diethyldithiocarbamic acid and 5 mM Na_2EDTA. After centrifugation at 12100 g for 15 min, the pellet was discarded and the supernatant was used for determination of the total antioxidant activity of the aqueous phase. Lipid extract was performed in the dark and under continuous flux of nitrogen using chloroform/methanol (2:1, v/v). The extract was washed three times with KCl 0.88% (w/v) in order to eliminate salts. Chloroform phases were taken to dryness with a rotavapor and resuspended in chloroform/ethanol (1:5, v/v). Soon after resuspension, lipid extract was used for determination of antioxidant activity of total lipid phase. The antioxidant capacity was measured on hydrophilic and lipophilic fractions of basil leaves and the total activity was calculated as the sum of the contribution of each fraction. Analysis was carried out according to Pellegrini et al. (1999) using the ABTS [2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)] method. The radical cation $ABTS^{+\cdot}$ solution was diluted in ethanol or water for lipid or aqueous extracts, respectively, in order to obtain an absorbance at 734 nm of 0.70 ± 0.05. After addition of the extract, the decrease in absorbance was monitored and compared to that of the trolox standard. Antioxidant activity was expressed in terms of trolox equivalent antioxidant capacity (TEAC)/g DW of plant material.

Phenolic acids

Leaf samples were added with 10 ml of extraction buffer (50% methanol, containing 1% HCl) and the extraction was carried out for 3 h in a boiling bath (150°C) under continuous stirring. After centrifugation at 12100 g for 15 min, the supernatant was collected and the extraction was repeated again twice on the pellet. The methanolic extracts were collected, vacuum dried and re-suspended in a solution containing 68% water, 30% acetonitrile and 2% acetic acid. Before analysis, the samples were passed through a Sartorius (Goettingen, Germany) filter (Minisart 0.45 mm) to remove any suspended material.

Qualitative and quantitative analysis were performed by a reverse-phase HPLC (Talcott and Howard, 1999). 20 µl were injected into a Waters model 515 HPLC system fitted with a 3.9 mm × 150 mm Nova-Pak C18 column (Waters, Milford, MA, USA). Detection was at 280 nm using a Waters 2487 dual λ UV-visible detector. Mobile phase A contained 98% water and 2% acetic acid, and mobile phase B contained 68% water, 30% acetonitrile, and 2% acetic acid. A linear gradient of 10 to 95% mobile phase B was run for 90 min at 1 ml min^{-1}. The identity of the phenolic acids was confirmed by co-chromatography on HPLC with authentic standards (Sigma Chemical Co., St. Louis, MO, USA), and quantification was performed using a standard curve in the range of 0.1 to 1 µg of standard mixtures containing gallic, *p*-hydroxybenzoic, vanillic, caffeic, syringic, *p*-coumaric, ferulic, gentisic and rosmarinic acid. Chromatogram analysis was performed by the software Millennium 32 (Waters).

Statistical analysis

The results are the means from three replicates from three

independent experiment (n = 9). All data are reported as mean values ± SE. The significance of differences among mean values was determined by one-way ANOVA. Comparisons among means were performed using Duncan's multiple range test. Reported means in tables and figures accompanied by different letters are significantly different at $P \leq 0.05$. When necessary, an arc sin or angular transformation was applied before statistical analysis was performed.

RESULTS

Antioxidant activity

In both Genovese and Fine leaves, hydrophilic extract had a higher antioxidant activity than the lipophilic one (Tables 1 and 2) and its contribution to TAA varied, after 15 days of treatment, from 67% (0 mM)-80% (25 mM Na_2SO_4) in Genovese cultivar to 59% (0 mM)-76% (25 mM Na_2SO_4) in Fine one and after 30 days from 58 to 74% (Genovese) to 64 to 84% (Fine), respectively under control and Na_2SO_4 salinity. However, the antioxidant activity, as measured by TEAC, was higher in Genovese than in Fine cultivar irrespective of the salt and the period of treatment (Tables 1 and 2).

In Genovese leaves, Na_2SO_4 treatment induced a significant increase in hydrophilic antioxidant activity (HAA) by 23 and 24% and in Fine ones by 77 and 19%, respectively after 15 and 30 days of treatment.

However, a decrease in lipophilic antioxidant activity (LAA) was monitored (Tables 1 and 2) in Genovese cultivar (-38% after 15 days and -42% after 30 days of treatment) and in Fine one (-17% after 15 days and -68% after 30 days of treatment). Moreover, TAA did not show any change in Genovese basil after 15 days of Na_2SO_4 treatment, while it showed a significant increase by 37% in Fine one after the same period of treatment. However, after 30 days of Na_2SO_4 treatment, TAA showed a low decrease by 4% in Genovese and by 12% in Fine cultivar.

Phenolic acids

Under Na_2SO_4 salinity, total phenolic acids, identified by HPLC, remained constant after 15 days and decreased significantly after 30 days by 42 and 67%, respectively in Genovese and Fine (Table 3). However, it is noteworthy that Fine cultivar contained the lowest content of phenolic acids. Main representative phenolic acids of *O. basilicum* leaves, cvs. Genovese and Fine were rosmarinic, gentisic and caffeic acids. All of them increased or remained constant with the age of basil (Figures 1A, B and 2A, B). In the Genovese cultivar, the content of caffeic and gentisic acids, after 15 days of treatment did not show variation with Na_2SO_4 salinity while that of rosmarinic acid decreased by 29% (Figure 1A). After 30 days, caffeic acid content remained constant with salinity whereas the content of rosmarinic and gentisic acids decreased by 45 and 43%, respectively (Figure 1B).

In the case of Fine cultivar, caffeic and rosmarinic acids contents decreased (-34 and -57%, respectively) after 15 days of treatment, however that of gentisic remained unchanged with Na_2SO_4 salinity (Figure 2A). After 30 days, the concentration of caffeic acids did not show variation but rosmarinic and gentisic acids contents decreased both by about 70% with salt (Figure 2B). Other phenolic acids identified in small amounts (about 10 to 100-fold less than the main phenolic acids) were gallic, coumaric, syringic, vanillic, p-OH-benzoic and ferulic acids. In the Genovese basil, the content of gallic, p-OH-benzoic, cumaric, ferulic and syringic acids, after 15 days of treatment did not show variation with Na_2SO_4 salinity while that of vanillic acid decreased by 50%.

After 30 days of treatment, p-OH-benzoic, vanillic and syringic acids contents significantly increased (+89, +50 and +113%, respectively) with salinity whereas the content of gallic, cumaric and ferulic acids remained constant (Figure 1C and D). In the case of Fine cultivar p-OH-benzoic, vanillic, cumaric and syringic acids contents after 15 days decreased by 75, 60, 50 and 67%, respectively, however that of gallic and ferulic remained unchanged with Na_2SO_4 salinity. After 30 days of treatment, the concentration of gallic and syringic acids did not show variation but p-OH-benzoic, cumaric and ferulic acids contents decreased (38, 33 and 44%, respectively), however that of vanillic acid increased by 31% with salt (Figure 2C and D).

DISCUSSION

Nutritionists, clinical researchers, and various segments of the food and pharmaceutical industries have an increasing need to know the antioxidant capacity of physiological fluids, foods, beverages and natural products because it is well-recognized that the role of antioxidant molecules is critical in the detoxification of free radicals (Halliwell, 2000). The nutraceutical properties of basil leaves are affected by the presence of two different types of antioxidants, the hydrophilic ones such as vitamin C, glutathione and phenolic acids and the lipophilic ones such as vitamin E and carotenoids, whose consumption is related to oxidative processes, which could be induced by environmental changes such as salinity. Indeed, Na_2SO_4 salinity stimulates the production of oxidizing agents in basil leaves and results in redox reactions leading to metabolic alterations and reduced basil leaves production (Tarchoune et al., 2010, 2012). Na_2SO_4 salt treatment may significantly improve the hydrophilic antioxidant activity of Genovese and Fine basil either after 15 or 30 days of treatment (Tables 1 and 2). Opposite patterns were observed for lipophilic antioxidant (Tables 1 and 2). With regard to the total antioxidant activity, after 15 days of treatment, Na_2SO_4

Table 1. Hydrophilic (HAA), lipophilic (LAA) and total antioxidant activity (TAA) extract from basil leaves (cv. Genovese), after 15 and 30 days of Na_2SO_4 treatment and determined by decolourisation of 2,20-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) radical cation ($ABTS^{+\cdot}$). TEAC, trolox equivalent antioxidant capacity.

TEAC (mmol g^{-1} DW)	15 days		30 days	
	Control	25 mM Na_2SO_4	Control	25 mM Na_2SO_4
HAA	53.4 ± 4.6^{c}	65.5 ± 2.5^{b}	64.0 ± 1.7^{b}	79.5 ± 0.8^{a}
LAA	25.9 ± 4.2^{b}	16.1 ± 3.7^{c}	47.2 ± 2.8^{a}	27.6 ± 1.6^{b}
TAA	79.4 ± 4.7^{c}	81.6 ± 1.3^{c}	111.2 ± 1.1^{a}	107.0 ± 1.2^{b}

Means (n = 3 ± SE) with different letters indicate significant differences at $P \leq 0.05$ between both salt and period of treatment as determined by Duncan's multiple range test.

Table 2. Hydrophilic (HAA), lipophilic (LAA) and total antioxidant activity (TAA) extract from basil leaves (cv. Fine), after 15 and 30 days of Na_2SO_4 treatment and determined by decolourisation of 2,20-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) radical cation ($ABTS^{+\cdot}$). TEAC, trolox equivalent antioxidant capacity.

TEAC (mmol g^{-1} DW)	15 days		30 days	
	Control	25 mM Na_2SO_4	Control	25 mM Na_2SO_4
HAA	33.7 ± 2.2^{c}	59.7 ± 3.8^{a}	48.1 ± 1.8^{b}	57.0 ± 2.4^{a}
LAA	23.7 ± 1.8^{b}	19.1 ± 1.5^{c}	26.7 ± 1.3^{a}	8.6 ± 0.3^{d}
TAA	57.4 ± 3.9^{c}	78.8 ± 5.3^{a}	74.8 ± 0.6^{a}	65.6 ± 2.6^{b}

Means (n = 3 ± SE) with different letters indicate significant differences at $P \leq 0.05$ between both salt and period of treatment as determined by Duncan's multiple range test

Table 3. Effect of Na_2SO_4 on total phenolic acids after 15 and 30 days of treatment in leaves of basil (*Ocimum basilicum* L.), cultivar Genovese and Fine.

	15 days		30 days	
	0 mM	25 mM Na_2SO_4	0 mM	25 mM Na_2SO_4
Genovese	14 ± 1.5^{b}	12 ± 1.4^{b}	26 ± 3.0^{a}	15 ± 4.2^{b}
Fine	7 ± 1.9^{b}	4 ± 1.1^{b}	18 ± 5.7^{a}	6 ± 1.5^{b}

Reported means followed by different letters are significantly different at $P \leq 0.05$ as determined by Duncan's multiple range test

salinity was less stressful for Fine cultivar (Table 2) that in such condition was able to increase its antioxidant power in comparison to Genovese one (Table 1). However after 30 days of Na_2SO_4 treatment, we observed a small decrease in the TAA (Tables 1 and 2) for both cultivars. Indeed, data published earlier (Tarchoune et al., 2009), using the same growth condition and NaCl as salt stressor suggest that the Fine cultivar was able to increase its antioxidant power appearing so more resistant to NaCl salinity in comparison to Genovese one.

Our two cultivars showed a different equipment of antioxidant compounds, in fact the Fine cultivar, which traditionally used as a ornamental herb had a lesser constitutive amount of hydrophilic and lipophilic antioxidants than the Genovese one (Tables 1 and 2). The latter is often present in Mediterranean diet and can be considered as good sources of natural antioxidants. Nguyen and Niemeyer (2008) and Sgherri et al. (2010) have found basil TEAC levels lower than those reported here (Tables 1 and 2). These discrepancies may be due to various extraneous reasons: agricultural and geographical conditions, different cultivars and age of harvests, as well as growth conditions. Together with phenolic compounds, ascorbic acid and glutathione represents the main water-soluble antioxidants of basil and contributes to the antioxidant activity of the water-soluble fraction. In the present work, although major phenolic acids in Genovese and Fine basil remained constant or decreased with salinity (Figures 1 and 2), we observed an increase in hydrophilic antioxidant power (Tables 1 and 2), this trend suggest that ascorbic acid and glutathione contributed to the antioxidative system of basil leaves. In fact, the increase in HAA was due to the increase in glutathione content after 15 days of treatment and in AsA content after 30 days either for Genovese

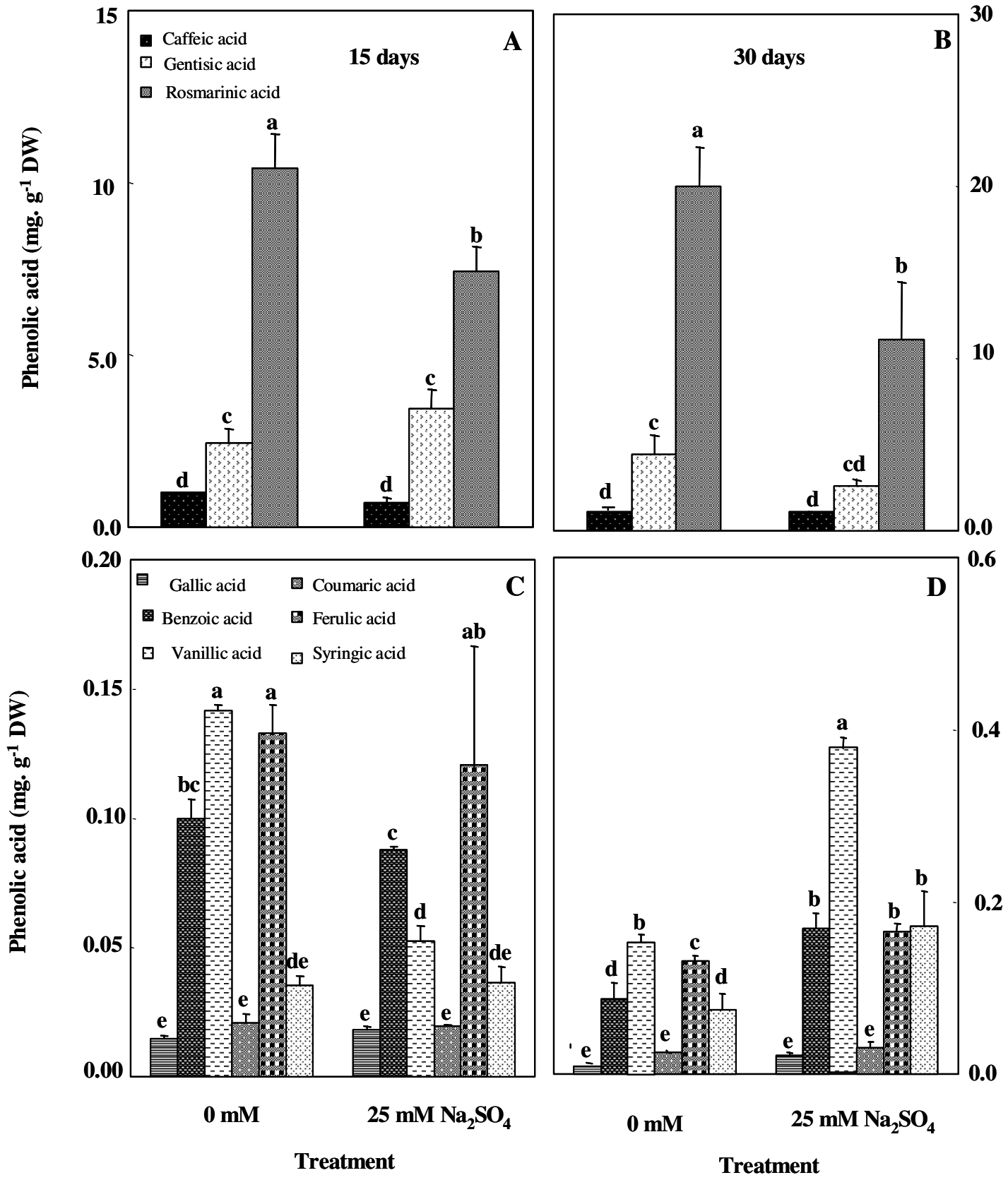

Figure 1. Most (A, B) and least (C, D) representative phenolic acids of *Ocimum basilicum*, cultivar Genovese subjected to 25 mM Na_2SO_4 for 15 (A, C) and 30 days (B, D). Reported means followed by different letters are significantly different at $P \leq 0.05$ as determined by Duncan's multiple range test.

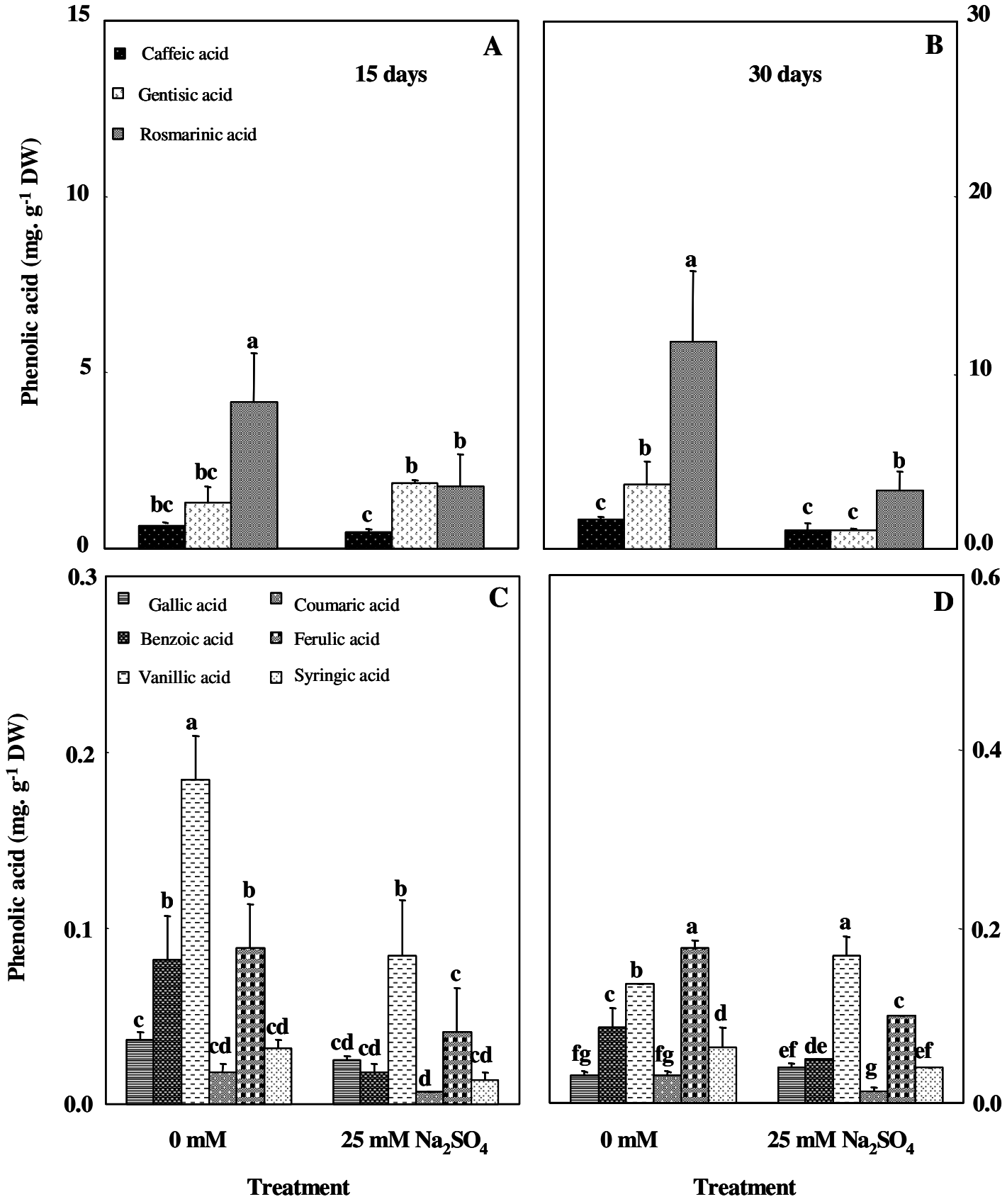

Figure 2. Most (A, B) and least (C, D) representative phenolic acids of *Ocimum basilicum*, cultivar Fine subjected to 25 mM Na_2SO_4 for 15 (A, C) and 30 days (B, D). Statistical analysis was as in Figure 1.

(Tarchoune et al., 2010) or Fine cultivar (Tarchoune et al., 2012). With regard to the basil lipophilic antioxidant, it decreased under Na_2SO_4 salinity (Tables 1 and 2), even if high values of tocopherol in both Genovese and Fine basil were observed (data not shown). Vitamin E is one of the most important antioxidants in the lipid phase; however, it is surprising how low its contribution was to the antioxidant activity of the lipophilic extracts of basil in comparison with other molecules such as chlorophyll (Sgherri et al., 2011). According to these authors, the contribution of tocopherols to the LAA was only of 0.3%. Moreover chlorophylls contribute to about 40% to the antioxidant activity of the lipid extract of basil. The lack of correlation between antioxidant compounds present in basil and total antioxidant activity could be explained by the fact that levels of single antioxidants do not necessarily reflect their total antioxidant activity (TAA); this depends on the synergic and redox interactions among the different molecules present in the basil.

Phenolic acids have repeatedly been implicated as natural antioxidants in fruits, vegetables, and other plants. For example, caffeic acid, ferulic acid, and vanillic acid are widely distributed in the plant kingdom (Larson, 1988). Rosmarinic acid, an important phytochemical, is known as an antiviral, antibacterial, antioxidant and anti-inflammatory agent (Klem et al., 2000; Jayasinghe, et al., 2003). The phenolic acids detected in Genovese and Fine basil (Figures 1 and 2) are in agreement with that previously found by Zgòrka and Glowniak (2001), Javanmardi et al. (2002) and Kivilompolo and Hyotylainen (2007). Rosmarinic acid was the predominant phenolic compound found in both basil cultivars (Figures 1A, B and 2A, B). The present results are in agreement with those by several authors who have reported that rosmarinic acid represents one of the most abundant forms of phenolic acids not only in *O. basilicum* (Zgòrka and Glowniak, 2001; Javanmardi et al., 2002; Shan et al., 2005), but also in *Salvia officinalis*, *Melilotus officinalis* and *Rosmarinus officinalis* (Zgòrka and Glowniak, 2001). Herbs from the family Lamiaceae, such as basil, rosemary, sage, and thyme, provide the only dietary source of rosmarinic acid (Crozier et al., 2006), with concentrations typically ranging from 2 to 27 mg/g DW (Wang et al., 2004). Although rosmarinic acid has been consistently noted as the predominant basil phenolic acid within the literature, in some basil cultivars rosmarinic acid is not the phenolic acid found in highest concentration (Lee and Scagel, 2010; Sgherri et al., 2010; Kwee and Niemeyer, 2011). Our results indicate that rosmarinic acid levels in basil were significantly affected by cultivar. In fact their concentrations ranged from 4 mg/g DW for Fine basil to 10 mg/g DW for Genovese one. However, the rosmarinic acid levels reported for basil in the literature vary widely: from 3.1 to 12.7 mg/g DW (Nguyen et al, 2010) and from 0.06 to 6.09 mg/g DW (Kwee and Niemeyer, 2011), are lower than concentrations previously determined for Iranian basil accessions, which ranged from 10 to 100 mg/g DW (Javanmardi et al., 2002).

High level of rosmarinic acid suggest that it might have a role in the scavenging of free radicals of oxygen under salinity conditions when oxidative stress increased (Tarchoune et al., 2009). In both basil and either after 15 or 30 days of treatment the decrease in rosmarinic acid, observed under Na_2SO_4 salinity (Figures 1A, B and 2A, B) might be related to a survival strategy of the plants which use their antioxidant power to oppose to the over noxious production of ROS.

The amount of most phenolic acids increased with growth but decreased under Na_2SO_4 salinity at both stage. This suggests a positive effect of growth on phenolic acids pool but, on the other hand, a negative impact of salinity on their content. Although the content of some minor phenolic acids such as p-OH-benzoic, vanillic and syringic acids (Genovese, after 30 days) and vanillic acid (Fine, after 30 days) increased with salinity, their levels remained very low. On the other side, although the contents of the most abundant phenolic acids decreased, their levels remained still high in comparison with the other phenolic acids, thus ensuring a certain reserve of these antioxidants and, explaining the stability of the total phenolic acids sum after 15 days of treatment. Moreover, salinity caused a marked reduction in the sum of phenolic acids in both cultivars after 30 days of treatment.(Table 3) The predominant oxidation of phenolic acids in *basil* can be due to increased activity of peroxidase (POD) under salt stress conditions (Tarchoune et al., 2010, 2012). Although some phenolic acids content increased during salt treatment, probably due to increased synthesis through activation of key enzymes in phenolic biosynthesis, such as phenlalanine ammonia-lyase (PAL), this was not enough and the sum of phenolic acids decreased.

In conclusion, the use of moderate salinity levels can be an effective method to produce basil of a superior nutritional value, which may compensate for the reduction in crop yield. This finding is a confirmation of what was previously found in basil under NaCl salinity (Tarchoune et al., 2009), even if the present research demonstrates how after 15 days of Na_2SO_4 treatment Fine and Genovese cultivars were able to increase or maintain unchanged their antioxidant power. Moreover, after 30 days, both cultivars decreased it. In fact, this capacity appears to be lesser under Na_2SO_4 salinity with respect to that under NaCl one (Tarchoune et al., 2009). The changes in phenolic acid contents could be of some importance for what concerns the nutritional value of basil: even if the major phenolic acid, rosmarinic acid, decreased upon salinity, their content remained very high. Despite the enormous interest in phenolic compounds as potential protective agents against the development of human diseases, the real contributions of such compounds to health maintenance and the mechanisms through which they act is still unclear.

Anyway, phenolic acids and, in particular, rosmarinic acid might have important biological effects for human health other than physiological roles in plants, and greater attention should be given to these compounds.

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