Ozone and Salinity Combined Stress Effects on Olive Leaf Antioxidant Enzyme Activities

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Abstract

The presence of high ambient ozone concentrations is common in the Mediterranean region, while saline water is often used for olive irrigation. The effects of this combined stress were studied in young olive trees. Two-year-old 'Konservolea' and 'Kalamata' olive plants grafted on olive seedlings were subjected to ambient O_3 levels or to charcoal-filtered air in open top chambers and irrigated with half strength Hoagland's solution with or without 100 mM NaCl during the growing period in 2006 and 2008. Superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) activities were measured periodically from July to October in this year's (young) and last year's (old) leaves. Enzyme activities increased gradually during summer followed by a sharp decrease in October, except APX in 2008, when the activity did not change significantly with time. This increase in antioxidant enzyme activities must be related to progressive stress during summer from high temperatures, salinity and ozone. Young leaves always had higher antioxidant enzyme activities than old leaves. 'Konservolea' leaves (especially the young ones) often had higher antioxidant enzyme activities than 'Kalamata' leaves, thus the former could be more resistant to salinity than the latter cultivar. The most photosynthetically active young leaves from both 'Konservolea' and 'Kalamata' were mainly affected by salinity. Old leaves from both cultivars were not significantly affected by either of the stresses.

INTRODUCTION

The olive tree is extensively cultivated around the Mediterranean area and irrigated olive orchards are highly productive. Groundwater aquifers in many coastal Mediterranean areas are often contaminated with sea water and saline water is often used for olive irrigation. The olive tree is considered a moderately salt tolerant crop (Maas and Hoffman, 1977; Al-Absi et al., 2003). Nevertheless, salinity has been found to cause stress and to negatively affect olive tree productivity (Chartzoulakis, 2005). Similarly, accumulated periods above 40 nl L^{-1} of ambient ozone, a highly oxidizing molecule, can also cause stress responses in plants. There is not much experience regarding the effects of ozone on olive trees.

It is well documented that, when plants are subjected to many environmental stresses including salinity, an overproduction of reactive oxygen species (ROS), including hydrogen peroxide, superoxide radical and hydroxyl radicals is induced. These compounds are thought to be responsible for the oxidative damage associated with plant stress (Mittler, 2002). Ozone acts in a similar manner. The enzymatic antioxidant system is the major stress protective mechanism in plants and includes superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) (Mittler et al., 2004). Resistance to stresses in a plant species is often associated with increased activities of these enzymes (Gara et al., 2003).

In an effort to understand the effects of the combined stress of salinity and ambient tropospheric ozone on olive plant, we monitored the activities of the major antioxidant enzymes under prolonged stress in leaves of young olive trees.

MATERIALS AND METHODS

Two-year-old uniform plants of two Greek table olive (*Olea europaea* L.) cultivars 'Konservolea' and 'Kalamata' grafted on seedling rootstock were planted in 12 L pots containing sand-perlite mixture 1:1 v/v. Plants of each cultivar were lightly pruned and transferred to open-top chambers (OTCs) described by Heagle et al. (1973). Each circular OTC had a diameter of 2.5 m, an open top diameter of 2 m and a height of 2.8 m. Each chamber was constructed of an iron frame covered with a HDPE sheet. Each OTC was continuously ventilated with an air ventilation unit bringing ambient outside air into the chamber at 1600 m^3 h^{-1}. Air was distributed via perforated tubes 15 cm in diameter positioned at a height of 70 cm along the chamber walls. Ambient or charcoal-filtered air fumigation and salinity treatment were applied from April until October for two experimental years, 2006 and 2008.

Four OTCs were used in this experiment, two chambers received charcoal-filtered air and the other two received ambient ozone. Half of the pots (randomly chosen) inside each chamber received two liters of 100 mM NaCl with half strength Hoagland's solution 2-3 times per week, while control pots received half strength Hoagland's solution. There were 4 pots per treatment in each chamber and from each cultivar.

To avoid salt shock, the high salinity treated plants were initially given 25 mM NaCl and the concentration increased in weekly intervals to 100 mM NaCl throughout April. The conductivity of the drainage water (leachate) was measured weekly to monitor salinity. In order to avoid salt accumulation in the pots, all pots were flushed with non saline water twice per month.

Ozone Monitoring

Ozone was monitored with two Eco Sensors ozone monitors (Model C-30ZX, Eco Sensors, Santa Fe, USA), one placed in a chamber with charcoal-filtered air and another one in a chamber with non-filtered (ambient) air. The data from each ozone analyzer were logged in a data logger and collected weekly.

Enzyme Extraction

On 5 July, 10 August, 8 September and 11 October, 2006, and on 11 July, 9 September, 13 October 2008, leaf samples were collected for enzyme analysis. Four plants of each cultivar and treatment in each chamber were used for sampling. Three leaves each of two different ages (last year's -old- leaves and newly developed -new- mature leaves) per plant were collected and placed in plastic bags. Two h later the leaves were dipped in liquid nitrogen, ground using a mortar and pestle and stored at -30°C. Frozen powdered leaf tissue (0.2 g) was homogenized in pre-chilled mortar and pestle with 2 ml of ice-cold 100 mM potassium phosphate extraction buffer (pH 7), containing 2% (w/v) PVPP, 1 mM EDTA, and 1 mM PMSF, while for APX extraction 1 mM ascorbic acid was added to the buffer. The homogenate was filtered through muslin cloth, centrifuged at 15000 g for 30 minutes at 4°C, and the supernatant was used as crude extract for enzyme activity assays. All steps for enzyme extraction procedure were carried out at 4ºC.

Enzyme Activity Assay

SOD activity was determined by measuring the amount of enzyme required to cause 50% inhibition of nitroblue tetrazolium chloride, as described by Giannopolitis and Ries (1977). Absorbance of the reaction mixture was read at 560 nm. CAT activity was determined by monitoring the disappearance of H_2O_2 by measuring the decrease in absorbance at 240 nm according to Abei (1983). APX activity was measured by the method of Nakano and Asada (1981), by measuring the decrease in absorbance of the oxidized ascorbate at 290 nm.

RESULTS AND DISCUSSION

Ozone levels in the ambient ozone fumigated chamber were above 40 nl L^{-1} every day and during all daylight hours all through the measurement period from mid May to mid October. Actual ambient ozone levels were close to 60-80 nl L^{-1} during daylight hours with accumulated AOT40 at 54176.5 from mid May to mid October. Mean monthly daylight ozone levels in the control chamber were between 62 and 73 nl L^{-1} and in the charcoal-filtered air chamber 14-20 nl L^{-1}.

Superoxide Dismutase (SOD) Activity

1. 2006 Data. Leaves from 'Konservolea' olive trees had significantly higher SOD activity than leaves from 'Kalamata' olive trees, but their actual values were relatively similar (Table 1). The difference between the two cultivars was mainly found in young leaves sampled late in the season (during the September and October measurements). SOD activity in 'Konservolea' leaves reached the highest values in September and dropped substantially in October (Table 1). This trend was found universally but dominantly in the salinity treatments. SOD changes over time were exactly the same for 'Kalamata' leaves except that the above trend was present in the ambient ozone treatment as well.

Salinity treatments increased SOD activity in 'Konservolea' leaves compared to control and ambient ozone treatments in July, August and September (Table 1). The opposite was true in October, when leaves from salinity treatments had lower SOD activity than leaves compared to those of the control and ambient ozone treatments. The same differences in treatments were found with 'Kalamata' leaves except in October, when leaves from salinity treatments had lower SOD activity than leaves mainly from the control trees. This year's (new) leaves had higher SOD activity than last year's (old) leaves in both cultivars and at all dates and treatments (Table 1).

2. 2008 Data. Leaves from 'Konservolea' olive trees had SOD activity similar to leaves from 'Kalamata' olive trees (Table 2). But young 'Konservolea leaves had higher SOD activity (especially late in season) than young 'Kalamata' leaves. SOD activity in 'Konservolea' leaves increased and reached the highest values in September and dropped substantially again in October (Table 2). This trend was found in all treatments but mainly in the salinity treatments. SOD changes over time were exactly the same for 'Kalamata' leaves. New leaves had higher SOD activity than old leaves in both cultivars and at all dates and treatments (Table 2). Salinity treatments increased SOD activity in 'Konservolea' leaves compared to control and ambient ozone treatments in July and September (Table 2). The opposite was true in October when leaves from salinity treatments had lower SOD activity than leaves from the control and ambient ozone treatments. The same differences in treatments were found with 'Kalamata' leaves except in October, when leaves from salinity treatments had lower SOD activity than leaves mainly from the control trees.

Catalase (CAT) Activity

1. 2006 Data. Leaves from 'Konservolea' olive trees had significantly higher catalase (CAT) activity than leaves from 'Kalamata' olive trees, but their actual values were relatively similar (Table 1). The difference between the two cultivars was mainly found in control leaves and in August. CAT activity in 'Konservolea' leaves from the control trees increased in August and remained unchanged until October, while, in leaves from the salinity treatments, CAT activity did not change over the summer but decreased in October and, in leaves from the ambient ozone treatment, did not change over the time period studied (Table 1). CAT activity in 'Kalamata' leaves from the control leaves increased only in October compared to summer levels, and, similarly to 'Konservolea', showed changes in leaves from the salinity and ambient ozone treatments (Table 1). Salinity treatments increased CAT activity in 'Konservolea' leaves compared to control and ambient ozone treatments in July, August and September (Table 1). The opposite was true in October when leaves from salinity treatments had lower CAT activity than leaves

from the control and ambient ozone treatments. The same differences in treatments were found with 'Kalamata' leaves (Table 1). New leaves had higher CAT activity than old leaves in both cultivars and at all dates and treatments (Table 1).

2. 2008 Data. Leaves from 'Konservolea' olive trees had similar CAT activity to leaves from 'Kalamata' olive trees despite the treatment, leaf age and time of leaf sampling (Table 2).

CAT activity in 'Konservolea' leaves from the control trees remained unchanged over the study period. In leaves from the salinity treatments, CAT activity did not change over the summer but decreased in October and, in leaves from the ambient ozone treatment, CAT activity transiently decreased in September and increased again at the July levels in October (Table 2). CAT activity in 'Kalamata' leaves from the control and ambient ozone treated trees transiently decreased in September and increased again in October, while, in leaves from the salinity treatments, CAT activity decreased in October. Salinity treatments increased CAT activity in 'Konservolea' leaves compared to control and ambient ozone treatments in July and September (Table 2). The opposite was true in October when leaves from salinity treatments had lower CAT activity than leaves from the control and ambient ozone treatments. The same differences in treatments were found with 'Kalamata' leaves (Table 2). New leaves had higher CAT activity than old leaves in both cultivars and at all dates and treatments (Table 2).

Ascorbate Peroxidase (APX) Activity

1. 2006 Data. Leaves from 'Konservolea' olive trees had similar ascorbate peroxidase (APX) activity to that of leaves from 'Kalamata' olive trees (Table 1). Leaves from 'Konservolea' olive trees had higher APX activity than leaves from 'Kalamata olive trees in young leaves and in October. There was no clear trend in leaf APX activity changes over time for the various treatments in both cultivars tested, but overall, in the leaves from salinity treatments, APX activity decreased in October and, in the leaves from control and ambient ozone treatments, increased in October or remained unchanged over time (Table 1). Salinity treatments increased APX activity in 'Konservolea' leaves compared to control and ambient ozone treatments in July, August and September and only compared to the control in October (Table 1). The same differences in treatments were found with 'Kalamata' leaves in July, August and September (Table 1), but in October, leaves from salinity treatments had higher APX activity than leaves mainly from the ambient ozone treatment. New leaves had higher APX activity than old leaves in both cultivars and at all dates and treatments (Table 1).

2. 2008 Data. Leaves from 'Konservolea' olive trees had similar ascorbate peroxidase (APX) activity to leaves from 'Kalamata' olive trees (Table 2), but leaves from 'Konservolea' olive trees had higher APX activity than leaves from 'Kalamata olive trees in young leaves and in October. There was no clear trend in APX activity changes over time in the various treatments in both cultivars tested, but overall, in the leaves from salinity plus ambient ozone treatment, APX activity decreased in October. In the leaves from salinity and no ozone, APX activity did not change over time and, in the leaves from control and ambient ozone treatments, it increased in October or remained unchanged over time (Table 2).

Salinity treatments increased APX activity in 'Konservolea' leaves compared to control and ambient ozone treatments in July and September and only compared to the control in October (Table 1). The same differences in treatments were found for 'Kalamata' leaves in July and September (Table 1), but in October, salinity treatments had higher APX activity than leaves mainly from the ambient ozone treatment. New leaves had higher APX activity than old leaves in both cultivars and at all dates and treatments (Table 2).

Based on the two years' results, leaves from 'Konservolea' trees often had higher enzyme activity than leaves from 'Kalamata' trees with differences especially found in leaves from this year's growth and late in the season. This probably means that the main antioxidant mechanism in 'Konservolea' trees is more active and this cultivar is more

tolerant to oxidative stress, in this case salinity stress, than 'Kalamata' olive, although actual differences were often small.

This year's (new) leaves always had higher enzymatic activity than last year's (old) leaves, since new leaves are more photosynthetically active and are exposed to sun more intensely and often over the course of a day. On the other hand, old leaves are closer to senescence and their reduced antioxidant enzyme activities may be interconnected to senescence and their sensitivity to oxidative damage due to age and shade (Prochazkova and Wilhelmova, 2007).

Leaf SOD activity had a maximum in September, a trend that was not found with the other two analyzed enzymes over the measurement period. This could indicate that SOD is a better stress indicator especially to high temperature and salinity stresses.

The salinity treatment significantly increased enzyme activities over the summer and September period and strongly reduced enzyme activities in October compared to the control. This reduction late in the season could be due to prolonged stress or due to leaf functioning failure (Goreta et al., 2007). Increased enzyme activity due to salinity is a major metabolic reaction to salinity stress found as demonstrated here in olives and, generally, is correlated to a plant's tolerance to salinity stress (Allen, 1995).

The ambient ozone levels did not have any effects on enzyme activity compared to the control. This means that these two olive cultivars are relatively resistant to prolonged exposure to high ambient ozone levels exceeding 60 nl L^{-1}. Sebastiani et al. (2002) showed that APX activity in olive leaves was not affected by ozone treatments in Frantoio, while it increased in Moraiolo plants exposed to 50 nl L^{-1} of ozone.

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Tables

Table 1. Antioxidant enzyme activities of this year's (N) and one year old (O) leaf extracts from 'Konservolea' and 'Kalamata' olive trees irrigated with ½ strength Hoagland's solution with charcoal filtered air (C) or ambient O_3 (NW) or 100 mM NaCl with ambient O_3 (WW) or with charcoal filtered air (WN) during the summer 2006. Differences due to sampling date, treatment and leaf age were all significant at $P<0.001$.

Date	Treat-ment	SOD (units/g FW)				CAT (units/g FW)				APX (units/g FW)			
		Konservolea		Kalamata		Konservolea		Kalamata		Konservolea		Kalamata	
		N	O	N	O	N	O	N	O	N	O	N	O
July	C	196	190	194	192	120	106	117	103	1.27	0.86	1.27	0.83
	WN	224	207	228	20	245	102	239	99	2.57	0.56	2.53	0.56
	WW	225	193	227	202	233	114	227	111	2.65	0.60	2.62	0.56
	NW	192	191	192	193	123	111	120	109	1.19	0.84	1.19	0.84
August	C	188	182	189	182	144	121	120	105	1.31	0.88	1.31	0.82
	WN	227	179	230	179	258	106	244	101	2.65	0.58	2.61	0.56
	WW	227	180	230	180	247	124	248	113	2.69	0.60	2.69	0.58
	NW	200	184	200	184	112	108	123	110	1.21	0.86	1.23	0.86
September	C	202	200	202	197	142	118	117	105	1.39	0.88	1.29	0.84
	WN	235	210	229	213	252	104	240	99	2.71	0.60	2.67	0.64
	WW	234	210	230	200	241	122	244	111	2.77	0.62	2.73	0.66
	NW	200	201	199	197	109	105	121	107	1.31	0.86	1.31	0.82
October	C	199	188	198	194	141	118	141	119	1.29	0.76	1.51	0.92
	WN	176	169	175	175	94	78	99	77	2.45	0.54	2.23	0.70
	WW	181	172	164	168	97	81	99	83	2.12	0.56	1.81	0.68
	NW	220	194	170	176	131	103	135	105	2.27	0.70	1.05	0.88
$LSD_{0.05}$		6.8		6.8		21.2		21.2		0.26		0.26	

Table 2. Antioxidant enzyme activities of this year's (N) and one year old (O) leaf extracts from 'Konservolea' and 'Kalamata' olive trees irrigated with ½ strength Hoagland's solution with charcoal filtered air (C) or ambient O_3 (NW) or 100 mM NaCl with ambient O_3 (WW) or with charcoal filtered air (WN) during the summer 2008. Differences due to sampling date, treatment and leaf age were all significant at $P<0.001$.

Date	Treatment	SOD (units/g FW)				CAT (units/g FW)				APX (units/g FW)			
		Konservolea		Kalamata		Konservolea		Kalamata		Konservolea		Kalamata	
		N	O	N	O	N	O	N	O	N	O	N	O
July	C	185	178	182	180	126	114	124	109	1.15	0.86	1.19	0.78
	WN	214	196	218	191	251	111	243	106	2.53	0.56	2.47	0.56
	WW	215	182	217	190	239	121	231	118	2.61	0.58	2.57	0.54
	NW	181	179	180	181	130	119	126	117	1.17	0.82	1.17	0.82
September	C	210	209	218	216	130	107	107	94	1.15	0.80	1.25	0.80
	WN	243	228	249	226	239	92	226	88	2.59	0.58	2.53	0.52
	WW	244	216	248	225	227	111	229	99	2.61	0.54	2.65	0.54
	NW	215	214	216	217	99	95	111	96	1.19	0.80	1.17	0.82
October	C	204	184	203	193	132	108	130	109	1.27	0.76	1.49	0.92
	WN	171	165	173	173	84	69	88	67	2.43	0.54	2.21	0.70
	WW	177	168	159	165	87	71	89	73	2.10	0.58	1.81	0.68
	NW	228	190	168	172	121	91	126	93	2.27	0.70	1.05	0.88
$LSD_{0.05}$		6.5		6.5		22.3		22.3		0.25		0.25	