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Sex-related differences of two ecologically divergent *Salix* species in the responses of enzyme activities to atmospheric CO_2 enrichment

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Abstract

Sex-related differences in the responses of plants to CO_2 enrichment are still rarely studied. In this study, we examined the effects of elevated atmospheric CO_2 (720 μmol mol^{-1}) on the activities of polyphenoloxidases (PPOs) and guaiacol peroxidases (PODs) in male and female plants of two ecologically divergent willow species *Salix repens* and *S. phylicifolia*. We detected that females invested more in PPO-based defence than did males, whose PPO activity decreased as a result of CO_2 enrichment. Moreover, we found that the inherently slow-growing *S. repens* had markedly higher POD activity than did the more rapid-growing *S. phylicifolia.* The PODs of these two species also differed in their biochemical properties.

Additional key words: peroxidase, polyphenol oxidase, willow.

Introduction

Concentration of CO_2 has increased markedly during the last few decades due to human activity, such as the burning fossil fuels, and changes in land use and deforestation, and the concentration is expected to double by 2100 (Intergovernmental Panel on Climate Change 2007). The effects of elevated CO_2 on the growth of plants are usually beneficial and plants grown under elevated CO_2 are often more resistant to various stresses than plants grown under ambient CO_2 concentration (Niinemets 2010).

In plants, reactive oxygen species (ROS) are generated during normal growth and development (Noctor and Foyer 1998). Enhanced atmospheric CO_2 is predicted to reduce the accumulation of ROS in chloroplasts due to the increased pCO_2/pO_2 ratio at the sites of photoreduction and the suppression of photorespiration in C_3 plants (Marabottini *et al.* 2001). A decrease in the basal level of ROS in response to an elevated CO_2 concentration may lead to depressed anti-oxidative defence in plants that are adapted to enhanced levels of CO_2 (Marabottini *et al.* 2001, Li *et al.* 2009, Gillespie *et al*. 2011).

Phenolic compounds are efficient antioxidants that are able to quench ROS. There are two types of enzymes that oxidise plant phenolics: peroxidases (PODs) and polyphenoloxidases (PPOs). Plant guaiacol peroxidases (class III peroxidases, EC 1.11.1.7) are heme-containing enzymes that oxidise a large variety of substrates. PODs use H_2O_2 as a co-substrate, thereby suppressing oxidative stress in plants. Due to their antioxidative properties, PODs are induced by many environmental stresses, *e.g*., by air pollution (Ruuhola *et al*. 2009). On the other hand, the pro-oxidative nature of PODs is related to their role in defence against biotic invaders including insect herbivores (*e.g*., Bi and Felton 1995, Barbehenn *et al*. 2010). PODs have also an important role in many physiological processes including lignification, suberin formation, and cross-linking of cell-wall proteins (Hiraga *et al.* 2001).

PPOs catalyse two types of reactions: the hydroxy-lation of monophenols to diphenols (EC 1.14.18.1) and the oxidation of *o*-diphenols to *o*-quinones (EC 1.10.3.1). PPOs are copper-proteins that utilise O_2 in the oxidation of phenolics. PPOs are localized in the chloroplast

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Abbreviations: POD - guaiacol peroxidase; PPO - polyphenoloxidase.
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thylakoid membranes (Trebst and Depka 1995) and are thought to quench photochemical oxidation in chloroplasts (Vaughn and Duke 1984). They are also important in defences against several classes of insect herbivores (*e.g*., Bi and Felton 1995, Ruuhola *et al*. 2008).

Willows, dioecious species belonging to the *Salicaceae* family, are known for a specific group of phenolic compounds, salicylates. The willow species used in this study are ecologically very divergent: *Salix phylicifolia* grows in fertile habitats, such as the banks of ditches, rivers, and lakes whereas *S. repens* grows in less fertile habitats, such as sandy shores or peatland meadows (Hämet-Ahti *et al*. 1998). The female and male plants of dioecious species are predicted to differ in their investments in vegetative growth and reproduction (Åhman 1997, Cornelissen and Stiling 2005, Nybakken *et al.* 2012). It is suggested that females invest more in reproduction and defence than do males whereas male plants are assumed to grow faster (Åhman 1997, Cornilessen and Stiling 2005).

Since the *Salix* family includes very divergent species that may respond to environmental changes differently, we used two ecologically and chemically different willow species. The purpose of our study was to investigate whether female and male plants differ in their responses to elevated atmospheric CO_2. We tested growth, water content, accumulation of sugars and proteins, and PPO and POD activities.

Materials and methods

Cuttings (approximately 15 cm) of 12 clones (6 females and 6 males) of *Salix phylicifolia* L. and 16 clones (8 females and 8 males) of *Salix repens* L. were collected in late May from eastern and western Finland, respectively. The cuttings were planted in mild-fertilised and limed peat (10:8:16 N:P:K, *Finnpeat*, *Kekkilä*, Oyj, Finland) in plastic pots (1.55 dm^3). The plants were watered daily and slightly fertilised every third week. The fertilisation of the plants was carried out as described in Häikiö *et al.* (2009) using N:P:K (19.0:4.4:20.2) fertiliser (*Kekkilä*).

The experiment was conducted in eight greenhouse-chambers (4 chambers/treatment), which were randomly assigned to two concentrations of CO_2: ambient CO_2 (control) and elevated CO_2 (CO_2 treatment). The CO_2 concentration in the ambient chambers was set to follow the variation out of doors (*ca*. 360 µmol mol^{-1}). Elevated CO_2 concentration was set to 720 µmol mol^{-1}. The CO_2 supply and control systems are described in detail in Zhou *et al.* (2012). Relative humidity (RH) was 55 ± 3 % in all the chambers (*TRH-302A*, Nokeval, Finland). The growth temperature and irradiance followed the natural variation. We placed one pot with two cuttings of each clone in each chamber. The duration of the experiment was 10 weeks.

The eight most representative clones were selected for the analyses: *S. phylicifolia* (4 females and 4 males) and *S. repens* (3 females and 5 males). For the measurements of enzyme activities, two mature leaves of *S. phylicifolia* and two to four mature leaves of *S. repens* were flash-frozen in liquid nitrogen and stored at -80 ºC prior to enzyme extraction. An additional set of mature leaves was collected and dried in a drying room (RH 10 %) for 24 h, after which the dry mass of the leaves was measured. Water content (WC) was calculated as % of fresh mass.

Crude enzyme fractions were prepared as reported previously in Ruuhola *et al.* (2011) with some modifications. A portion of 0.25 g of polyvinylpolypyrrolidone (PVPP) (*Sigma*, St. Louis, USA) and 0.20 g of ground leaf material was weighed into an extraction vial (6 cm^3), and 5 cm^3 of ice cold extraction buffer [1 mM PMSF (*Sigma*), 1 mM dithiotreitol (*Sigma*), 1 % (v/v) Triton-X100 in 0.1 M potassium buffer, pH 7.5]. The samples were homogenised with a *Percellys® 24* homogenizator (*Bertin Technologies,* France) at 6 500 rpm for 60 s. The homogenates were shaked at 100 rpm with a plate shaker (*Infors AG*, *Berner Laboratorio-laitteet*, Bottningen, Finland) in an ice bath for 30 min. The samples were centrifuged at 2 150 *g* and 4 °C for 20 min (*A-4-62* rotor, *Eppendorf® 5810R* centrifuge, Hamburg, Germany). The supernatants were filtered through cheesecloth and the samples were stored at -80 °C prior to enzymatic assays.

Polyphenoloxidase (PPO) and peroxidase (POD) activity measurements were based on the methods presented in Ruuhola *et al.* (2011). PPO activity was measured with 120 mM catechol as a substrate in a 0.1 M potassium phosphate buffer at pH 6.8, and the rate of absorbance change at 420 nm was followed for 3 min with a spectrophotometer (*Cary 50 bio* UV-VIS spectrophotometer, *Varian*, Mulgrave, Australia). POD activity was measured with 100 mM (*S. repens*) and 120 mM (*S. phylicifolia*) guaiacol as a substrate and 65 mM H_2O_2 as a co-substrate in a 0.1 M K-phosphate buffer at pH 6.0 (*S. repens*) and 6.2 (*S. phylicifolia*), and the rate of absorbance change at 470 nm was followed for 3 min. Optimal conditions for PPO activities were determined by measuring the activity with various substrate concentrations (20 - 140 mM). PPO activity as a function of pH was determined over a pH range of 5.8 - 7.2. Optimal substrate concentrations for POD activities were determined by measuring the activity with various guaiacol concentrations: 40 - 120 mM for *S. repens* and 60 - 140 mM for *S. phylicifolia*. POD activity as a function of pH was determined over a pH range of 5.8 - 6.8. The effects of ascorbic acid (AA) on PPO and POD activities were determined by measuring the activities over a range of 0 -120 mM AA. All the activities were measured in duplicate. One unit was the amount of enzyme required to increase the absorbance of 0.001 per min. The protein content of the enzyme extract

was measured in duplicate with the spectrophotometer at 595 nm using the *BioRad* (Hercules, USA) protein assay method based on the Bradford (1976) method. Bovine serum albumin was used as a standard.

The data were analysed by mixed model (*SPSS 17.0*) where treatment, sex, and the interaction between them were set as fixed factors, with treatment chamber nested within treatment and its interaction with plant sex being random factors. Clone (genotype) was nested under sex, and this term was set as a fixed factor. Genotype was included in the model in order to analyse its main effect. Contrasts were tested by the least significance difference (LSD) method. When necessary, the data were normalised using an *ln*-transformation. The normality of data was checked by Kolmogorv's test and Q-Q plot and the homoscedasticity of variances was tested by Levene's test. Differences in the measured parameters between species were tested by the Kruskall-Wallis non-parametric test, due to the heteroscedasticity of variances when both species were included in the test.

Results

Specific PPO activity was higher in *Salix phylicifolia* than in *S. repens* whereas all POD activities (per f.m., d.m., and protein) were markedly higher in *S. repens* (Table 2). PPO activity was not affected by CO_2 treatment in either of the species whereas sex had a significant effect on the PPO activity of *S. repens* (Table 1). Males of *S. repens* had lower PPO activity (per f.m. or d.m. basis) than did females but this difference was significant only in the control treatment (Table 2). The POD activity of *S. repens* was affected both by the CO_2 treatment and by sex (Table 1). The POD activity of male plants (per f.m. and d.m. bases) was reduced by CO_2 enrichment and males also had lower POD activity than did females (Table 2). The POD activity of *S. phylicifolia* was not affected by CO_2 enrichment whereas the sexes differed in their POD activities (Table 1), males again having lower POD activity than females (Table 2).

Leaf protein content was higher in *S. repens* than in *S. phylicifolia* (Table 2). The CO_2 treatment had no significant effect on protein content, although in both species, it tended to decrease as a result of CO_2 enrichment (Table 2). Males of *S. repens* had lower protein content than females, but this difference was significant only for the control plants (Table 1, 2). Water content of leaves was higher in broad-leafed *S. phylicifolia* than in *S. repens*, a species of more arid habitats (Fig. 1*H*). Water content of *S. phylicifolia* decreased as a result of CO_2 enrichment but only in the female plants (Tables 1, 2). Consequently, *S. phylicifolia* males had higher water content in their leaves than did females in the CO_2 treatment (Table 2). Leaf water content of *S. repens* was not affected by CO_2 enrichment or by plant sex (Table 1). *S. phylicifolia*, as a species of moist habitats, had markedly higher leaf mass than *S. repens*, an inherently slow-growing species (Table 2). Leaf mass of female *S. phylicifolia* plants was increased by CO_2 enrichment whereas that of males was not affected (Table 2). Leaf mass of *S. repens* was affected neither by CO_2 treatment nor by plant sex (Table 1).

S. repens genotypes differed in their POD activities whereas *S. phylicifolia* genotypes differed in their PPO activities (Table 1). The genotype had a significant effect

Table 1. The effects of plant sex and CO_2 enrichment on the activities of PPOs and PODs, protein and water content, and dry mass of the leaves of *Salix repens* and *S. phylicifolia* analyzed with mixed model (*P* - values; *SPSS 19.0* for *Windows*). Significant cases ($P \leq 0.05$) are marked by *asterisks*, $n = 28$ - 39.

Species	Parameter	Sex (S)	Treatment (T)	S × T	Clone × S
S. repens	PPO [U mg^{-1}(f.m.)]	0.016*	0.889	0.397	0.525
	PPO [U mg^{-1}(d.m.)]	0.031*	0.565	0.550	0.109
	PPO [U mg^{-1}(protein)]	0.700	0.139	0.309	0.134
	POD [U mg^{-1}(f.m.)]	0.009*	0.032*	0.654	<0.001*
	POD [U mg^{-1}(d.m.)]	0.022*	0.008*	0.191	<0.001*
	POD [U mg^{-1}(protein)]	0.176	0.568	0.288	<0.001*
	protein content [mg g^{-1}(d.m)]	0.008*	0.136	0.168	0.148
	water content [%]	0.186	0.172	0.635	<0.001*
	leaf mass [mg(d.m.)]	0.538	0.623	0.634	<0.001*
S. phylicifolia	PPO [U mg^{-1}(f.m.)]	0.259	0.730	0.141	0.083*
	PPO [U mg^{-1}(d.m.)]	0.447	0.789	0.317	0.031*
	PPO [U mg^{-1}(protein)]	0.658	0.148	0.383	0.721
	POD [U mg^{-1}(f.m.)]	0.007*	0.903	0.843	0.183
	POD [U mg^{-1}(d.m.)]	0.010*	0.929	0.768	0.180
	POD [U mg^{-1}(protein)]	0.010*	0.587	0.608	0.286
	protein content [mg g^{-1}(d.m)]	0.410	0.685	0.994	0.597
	water content [%]	<0.001	0.043	0.046	<0.001*
	leaf mass [mg(d.m.)]	0.106	0.029	0.196	0.008*

Table 2. The effects of plant sex and CO_2 enrichment on the activities of PPOs and PODs, protein and water content, and dry mass of the leaves of *Salix repens* and *S. phylicifolia.* Values represent means ± SE, n = 28 - 39. Statistically significant differences between treatments are marked with different letters and between sexes with stars (* - $P \leq 0.05$; ** - $P \leq 0.01$; ***$P \leq 0.001$).

Species	Parameter	Ambient CO_2 females	Ambient CO_2 males	Elevated CO_2 females	Elevated CO_2 males
S. repens	PPO [U mg^{-1}(f.m.)]	7.306±0.667	5.462±0.541*	6.929±0.639	6.025±0.529
	PPO [U mg^{-1}(d.m.)]	21.86±2.19	16.78±1.75*	19.57±2.01	16.63±1.65
	PPO [U mg^{-1}(protein)]	1141±139	1321±115	1468±133	1386±113
	POD [U mg^{-1}(f.m.)]	50.00±14.7	29.81±6.64[a]	33.52±9.31	16.30±3.63[b]*
	POD [U mg^{-1}(d.m.)]	123.3±29.3	100.1±19.6[a]	92.46±21.7	44.39±8.33[b]**
	POD [U mg^{-1}(protein)]	10020±1700	9570±1330	10770±1620	7100±1330
	protein content [mg g^{-1}(d.m)]	21.08±2.20	13.20±2.00**	15.58±2.00	12.98±1.60
	water content [%]	64.46±1.57	66.62±1.48	62.30±1.45	63.31±1.34
	leaf mass [mg(d.m.)]	22.64±1.68	24.19±1.34	22.52±1.60	22.72±1.27
S. phylicifolia	PPO [U mg^{-1}(f.m.)]	7.282±1.13	7.415±0.932	8.945±0.951	6.379±0.919
	PPO [U mg^{-1}(d.m.)]	25.22±4.03	25.55±3.33	27.61±3.39	21.44±3.28
	PPO [U mg^{-1}(protein)]	2386±286	2478±250	3018±255	2682±246
	POD [U mg^{-1}(f.m.)]	2.960±1.76	0.676±0.334*	2.869±1.45	0.770±0.375*
	POD [U mg^{-1}(d.m.)]	10.19±6.26	2.284±1.17*	8.671±4.53	2.489±1.26
	POD [U mg^{-1}(protein)]	9 08.7±490	235.1±107	971.7±452	357.1±161
	protein content[mg g^{-1}(d.m)]	11.31±2.45	9.422±1.75	8.900±1.69	6.88±1.26
	water content [%]	70.31±0.601[a]	70.33±0.622	65.91±0.601[b]	68.66±0.622**
	leaf mass [mg(d.m.)]	105.8±7.66	130.4±8.20	136.2±7.66	139.1±7.93

on water content and leaf mass in both species (Table 1).

PPOs acted well in a wide range of pH, pH 6.8 being optimal for *S. phylicifolia* (Table 3). In *S. repens,* the highest PPO activity was detected between pH 6.4 and 6.8 (Table 3) . The POD activity of *S. repens* was rather steady within a pH range from 5.8 to 6.8 (Table 3). By contrast, the POD activity of *S. phylicifolia* varied with pH and two clear activity peaks were detected at pH 6.2 and 6.6 (Table 3). The PPO activity of *S. phylicifolia* was inhibited by ascorbic acid (AA), the difference being 46 % between 0 mM and 120 mM AA whereas the PPO activity of *S. repens* was not markedly inhibited by AA (Table 3). The POD activity of *S. repens* was, however, almost totally inhibited by 2.5 mM AA whereas the POD activity of *S. phylicifolia* was inhibited by 50 % in the presence of 120 mM AA (Table 3).

Discussion

The studied willow species and sexes differed in their responses to elevated CO_2. POD activities were reduced by elevated CO_2 in *S. repens* males whereas water content decreased and leaf mass increased in female plants of *S. phylicifolia*. In general, male willows had lower PPO activities than did female willows. In an earlier study, we found that PPO activity and content of salicylates, important defences of willows against herbivores, decreased in male *S. myrsinifolia* in response to elevated UV radiation (Ruuhola *et al*., unpublished results). Males had a lower content of chlorogenic acid, a substrate of PPOs and PODs, than had female *S. myrsinifolia*.

These results support earlier studies showing that female willows are better defended than male willows (Danell *et al.* 1985, Hjältén 1992, Cornilessen and Stiling 2005) since PPOs and PODs are important defences against both biotic and abiotic stressors. However, these results obtained with *Salix* species contradict the results obtained with *Populus* species, whose females were more

Table 3. PPO and POD activities of *S. repens* and *S. phylicifolia* as affected by different pH and by the concentration of ascorbic acid (AA) [mM], a PPO inhibitor.

	PPO [U mg^{-1}(protein)]			POD [U mg^{-1}(protein)]	
pH	*S. repens*	*S. phylicifolia*	pH	*S. repens*	*S. phylicifolia*
5.8	2191	3185	5.8	8824	2657
6.0	1742	2523	6.0	9020	3823
6.4	2630	2404	6.2	8591	6891
6.8	2610	3611	6.4	7577	4608
7.2	2004	2819	6.6	8044	6666
			6.8	7210	1263
AA			AA		
0	1533	5420	0	26452	5065
20	1808	3773	2.5	1650	-
60	2067	3486	5	1046	-
120	1285	2719	20	-	1903
			60	-	1420
			120	-	1207

sensitive to environmental (*e.g*., Xu *et al.* 2008, Zhang *et al.* 2012) and also biotic (Zhang *et al*. 2010) stresses than males. Moreover, male poplars also exhibited higher PPO and POD activities than female poplars (Xu *et al.* 2008, Zhang *et al.* 2012) which is contradictory to what we detected in willows.

It is predicted that an increase in atmospheric CO_2 will reduce the accumulation of basal-level ROS in plant leaves leading to a concomitant decrease in antioxidative defence in plants (Schwanz and Polle 1998, Marabottini *et al.* 2001, Gillespie *et al*. 2011). A similar decrease in ROS-scavenging enzymes to that which we detected in *S. repens* has been detected in several plant species (*e.g*., Schwanz and Polle 1998, Li *et al.* 2009 Ye *et al.* 2010). However, the results on how elevated CO_2 affects the antioxidative defence of plants have been variable depending on the species, the specific antioxidant, and also on the season (Schwanz and Polle 1998, Lavola *et al.* 2000, Marabottini *et al.* 2001, Tegelberg *et al.* 2008, Li *et al.* 2009).

The decrease in amount of antioxidants may theoretically depress the antioxidative defence of plants under stresses associated with global change (Marabottini *et al*. 2001). There is, however, evidence that plants grown under elevated CO_2 have a better ability to defend themselves against ozone (*e.g*. Peltonen *et al*. 2006, Gillespie *et al*. 2011) and UV-B (Lavola *et al*. 2000). In addition, plants grown under elevated CO_2 are often better defended against herbivores (Lindroth *et al.* 1995, Veteli *et al.* 2002, Niinemets 2010, Ye *et al.* 2010).

Leaf dry mass was increased as a response to CO_2 enrichment only in the fast-growing *S. phylicifolia* but not in the slow-growing *S. repens*. This might be related to the low sink capacity of *S. repens* which limits photosynthesis (see Li *et al.* 1999). The predominant effect of CO_2 enrichment is an increase in the photosynthetic rate leading to the accumulation of non-structural saccharides (Poorter *et al.* 1997, Ibrahim and Jaafar 2012), which may explain the increase in the leaf dry mass of *S. phylicifolia*. This accumulation of saccharides, probably also of phenolics (Ibrahim and Jaafar 2012), and non-significant depression in the protein content may explain the significant decrease in the water content that was observed in *S. phylicifolia*. However, we did not measure the photosynthesis rate, thus we cannot confirm this possibility.

A decrease in nitrogen and water content is regularly observed in boreal trees and shrubs as a response to CO_2 enrichment (Veteli *et al.* 2002, Stilling and Cornelissen 2007, Peltonen *et al.* 2010). However, an enhanced development and phenology of trees grown under CO_2 enrichment may also explain the decrease in protein and water content (*e.g*. Kellomäki and Wang 2001, Veteli *et al.* 2002, Tegelberg *et al.* 2008). Enhanced development could also explain the suppression of POD activity, since POD activity markedly decreases with leaf development (Yang *et al.* 2007).

In the resource availability theory, it is predicted that species of resource-poor environments grow inherently more slowly but invest more in constitutive defences and suffer lower from herbivore damage than do species adapted to more fertile sites (Coley *et al*. 1985, Endara and Coley 2011). Our results with *Salix* species at least partially support this assumption. The substantially higher POD activity in the leaves of *S. repens*, a species indigenous to resource-poor environments, than in the leaves of *S. phylicifolia*, a species of resource-rich environments, may also indicate a better ability to resist natural enemies since PODs have also a function in defence against herbivores (*e.g*. Barbehenn *et al*. 2010). In addition, *S. repens* leaves contain tremulacin and salicortin, which are toxic to herbivores (Ruuhola *et al*. 2003), whereas *S. phylicifolia* contains a low level or totally lacks salicylates (Ruuhola *et al*. 2001).

The POD enzymes of the two willow species deviated in their characteristics. The POD activity of *S. repens* was rather steady within a studied pH range from 5.6 to 6.8. By contrast, two separate guaiacol oxidation activity peaks with distinct pH optima (pH 6.2 and 6.6) were found in *S. phylicifolia* indicating that the guaiacol oxidation was accomplished by different isoenzymes. The more alkaline peak may represent a laccase type of enzyme activity. This is supported by the fact that we detected 20 % POD activity in *S. phylicifolia* without H_2O_2 whereas no activity was found in *S. repens* (data not shown). The laccase type of enzymes (*p*-diphenol:oxygen oxidoreductase, EC 1.10.3.2) catalyse, similarly to PODs but independently from H_2O_2, the oxidation of coniferyl alcohol, and laccases are thought to function in the biosynthesis of lignin (Ranocha *et al*. 2002).

PPO activities *in vivo* are also affected by the concentration of endogenic substrates (phenolics), pH, and reducing agents, such as AA (see Bi and Felton 1995). It is suggested that PODs act as an efficient H_2O_2 scavenging system in plant vacuoles in the presence of phenolics and AA (Takahama and Oniki 1997). Phenolic oxidation produces semiquinone radicals consuming H_2O_2, and semiquinone radicals are reduced back to parental phenolics by AA (Takahama and Oniki 1997). We found that the POD of *S. repens* was efficiently inhibited by AA suggesting that a similar H_2O_2 detoxifying system may exist in willows. POD oxidises phenolics to quinones by a one-electron transfer mechanism producing first semiquinone radicals that can be reduced back to parental phenolics by AA or they can be further oxidised to quinones, whereas PPO catalyses a two-electron transfer reaction producing quinones directly. We detected only a partial reduction in PPO (and POD) activity in *S. phylicifolia* whereas the PPO activity of *S. repens* did not show any inhibition by AA. Thus, we suggest that AA functions specifically in reducing the semiquinone radicals produced by PODs. Interestingly, POD activity was up-regulated in ascorbate (AA) deficient mutants of *Lathyrus sativus*, and these AA deficient plants were able to compensate low AA level with another type of antioxidants (Talukdar 2012).

We found that the two willow species and genders differed in their investments in PPO-based defence and also in their responses to CO_2 enrichment. Males had lower PPO activities than did females and these were further reduced by CO_2 enrichment supporting the assumption that male willows are less-defended than female willows. We also found that *S. repens*, a species of resource-poor environments, had markedly higher POD activity than *S. phylicifolia*, a species of resource-rich environments, supporting the resource availability hypothesis. The inherently slow-growing *S. repens* responded to the CO_2 enrichment by reducing its POD activity whereas the more rapid-growing *S. phylicifolia* responded by increasing the leaf dry mass and reducing the water content of its leaves. These changes may be related to the advanced phenology of plants grown under CO_2 enrichment.

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