Postharvest Biology and Technology 60 (2011) 149–157

Contents lists available at ScienceDirect

Postharvest Biology and Technology

journal homepage: www.elsevier.com/locate/postharvbio

Inhibitors of oxidative enzymes affect water uptake and vase life of cut *Acacia holosericea* and *Chamelaucium uncinatum* stems

Fisun G. Çelikel[a,b,*], Daryl C. Joyce[a], John D. Faragher[a]

[a] The University of Queensland, Centre for Native Floriculture, School of Land, Crop and Food Sciences, Gatton, QLD 4343, Australia
[b] Ondokuz Mayis University, Faculty of Agriculture, Department of Horticulture, 55139 Kurupelit, Samsun, Turkey

ARTICLE INFO

Article history:
Received 21 August 2010
Accepted 18 December 2010

Keywords:
Acacia
Chamelaucium
Inhibitors
Peroxidase
Phenoloxidase
Physiological occlusion
Water uptake
Wound reaction

ABSTRACT

Cut *Acacia holosericea* (Velvet Leaf Wattle) foliage has a short vase life, possibly because of blockage in xylem vessels. We indirectly investigated a hypothesised role for peroxidase and phenoloxidase enzyme activities in xylem occlusion of Acacia stems by using their inhibitors. We also tested these inhibitors with cut *Chamelaucium uncinatum* (Geraldton waxflower), another woody stemmed cut flower.

The peroxidase inhibitors used were 3-amino-1,2,4-triazole (AT), catechol (CH), hydroquinone (HQ), p-phenylene diamine (PD) and copper sulphate (CS, Chamelaucium only). The catechol oxidase inhibitors were tropolone (TP), 4-hexylresorcinol (HR) and 2,3-dihydroxynaphthalene (DN). A laccase inhibitor, cetyltrimethylammonium bromide (CM), was also used. Other phenoloxidase inhibitors tested were p-chlorophenol (CP), p-nitrocathechol (NC), p-nitrophenol (NP) and sodium metabisulphite (SM). 2-Mercaptoethanol (ME), phenyl hydrazine (PH) and salicylhydroxamic acid (SH) were used as inhibitors of both peroxidase and phenoloxidase.

Twelve inhibitors (CH, HQ, DN, HR, TP, CM, CP, NC, NP, SM, PH and SH) significantly improved water uptake, maintained relative fresh weight and increased vase life of Acacia at least once in two experiments. In Chamelaucium, six inhibitors had significant positive effects on water relations (CH, PD, CS, CM, CP and PH) and vase life (AT, CH, PD, CS, ME and PH), while four of them (DN, TP, NC and NP) were phytotoxic at applied concentrations. Only one of the 46 inhibitor treatments inhibited transpiration and increased fresh weight, suggesting that the inhibitors mainly acted by increasing water uptake. Overall, results indicate that oxidative enzyme activities, potentially through phenolic deposition, contribute to xylem occlusion in woody cut stems of Acacia and Chamelaucium.

1. Introduction

Acacia holosericea (Velvet Leaf Wattle) has cut foliage potential due to its silvery green silky phyllodes (leaves). However, it has a short vase life due to insufficient water uptake causing early wilting and/or desiccation of phyllodes (Damunupola et al., 2010). Water uptake by cut flower stems, including *A. holosericea*, decreases with time after being stood into water, possibly due to occlusions in the xylem (Van Doorn, 1996). Physical blockages of xylem due to air embolism (cavitation), small particles or bacteria and bacterial products are well known (Van Doorn, 1996). Physiological plugging as a result of defensive metabolic responses to wounding has also been reported (Van Doorn and Cruz, 2000; Van Doorn and Vaslier, 2002; Vaslier and van Doorn, 2003; Loubaud and van Doorn, 2004; He et al., 2006).

Wounding plant tissues activates mechanisms for defence and healing (Leon et al., 2001). Deposition of materials such as suberin, lignin and tannin in the xylem as a physiological reaction to wounding was suggested long ago (Dean and Kolattukudy, 1976; Halevy and Mayak, 1981; Van Doorn, 1996). Williamson et al. (2002) considered that hydrophobic suberin formation was an early (<12 h) response to wounding that led to cavitation and water deficit stress. Peroxidase is involved in suberin deposition in wound healing potato tuber tissue (Espelie et al., 1986). The wound signal is transmitted over a distance of at least 4 cm through pea stem tissue (Peck and Kende, 1998). With no delay in response intensity, a hydraulic or electric signal is possibly responsible for spread of the wound response.

Inhibition by antioxidants of wound-induced xylem occlusion has been reported to occur in Chrysanthemum both during dry and wet storage (Van Doorn and Cruz, 2000). Catechol oxidase and laccase are major phenoloxidases (Walker, 1995). Xylem blockage in

* Corresponding author at: Ondokuz Mayis University, Faculty of Agriculture, Department of Horticulture, 55139 Kurupelit, Samsun, Turkey.
Tel.: +90 362 3121919x1122; fax: +90 362 4576034.
E-mail addresses: fgcelikel@omu.edu.tr, fgcelikel@gmail.com (F.G. Çelikel).

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doi:10.1016/j.postharvbio.2010.12.009

Chrysanthemum was delayed by the catechol oxidase inhibitors, tropolone and 4-hexylresorcinol (Van Doorn and Vaslier, 2002). Both peroxidase and catechol oxidase were involved in physiological blockage in the lowermost 5 cm of dry stored Bouvardia flower stems (Vaslier and van Doorn, 2003). Similar physiological blockage also occurs in Astilbe flowers (Loubaud and van Doorn, 2004).

S-carvone is a monoterpene known to inhibit phenylalanine ammonia-lyase (PAL) activity and synthesis of wound-induced compounds, like suberin (Oosterhaven et al., 1995a). However, it also has antimicrobial activity (Oosterhaven et al., 1995b). In vase solution, S-carvone extended the vase life of flowers from members of the families Proteaceae [viz. *Hakea francisiana* (Williamson et al., 2002) and *Grevillea* 'Crimson Yul-lo' (He et al., 2006)] and Myrtaceae [viz. *Baeckea frutescens* and *Chamelaucium uncinatum* (Damunupola et al., 2010)]. However, there were no positive effects on vase life of *A. holosericea* and Chrysanthemum (Damunupola et al., 2010). Like S-carvone, treatment with a catechol oxidase inhibitor, 4-hexylresorcinol, delayed stem end blockage in *Grevillea* 'Crimson Yul-lo' inflorescences (He et al., 2006). Thus, blockage in cut Grevillea stems was suggested to be physiologically mediated.

Peroxidase, polyphenoloxidase and PAL activities after wounding are associated with lignin and phenol metabolism (Okey et al., 1997). As a rapid response to wounding in wheat roots, peroxidases from cell surface release into the apoplast where they can display both oxidative and peroxidative activities (Minibayeva et al., 2009).

In the present study, inhibitors of peroxidase and phenoloxidase (viz. catechol oxidase, laccase, etc.) enzymes were tested for woody *A. holosericea* and *C. uncinatum* stems. It was hypothesised that these inhibitors of oxidative enzymes would suppress enzyme activity in response to wounding and thereby prevent or reduce physiological occlusion of xylem vessels for these species.

2. Materials and methods

2.1. Plant materials

Leafy stems of *A. holosericea* A. Cunn. ex G. Don (Velvet Leaf Wattle, Family Mimosaceae) around 42–55 cm long were harvested in mornings over winter from young (1-year-old; experiment 1; 30 June 2009) and established (3–4-year-old; experiment 2; 14 July 2009) plants growing at The University of Queensland Gatton campus nursery (152°20′ E, 27°33′S). Flowering stems of *C. uncinatum* Schau., Myrtaceae 'Dancing Queen' (Geraldton waxflower; Family Myrtaceae) around 30–35 cm long were harvested in the morning in winter (30 July 2009) from mature stock plants at the same nursery. Stems were transported dry to the laboratory within 1 h of harvest. Phyllodes for Acacia and leaves for Chamelaucium were removed from the lowermost stem. Within 2 h of harvest, stems were recut to 40 and 25 cm for Acacia and Chamelaucium, respectively, under deionised water (DI) immediately before placing them into pre-prepared pulsing solutions.

2.2. Inhibitor treatments

Fifteen compounds (16 for Chamelaucium) known to inhibit peroxidase and/or phenoloxidase enzyme activities were tested (Table 1). The inhibitors of peroxidase were 3-amino-1,2,4-triazole (amitrol, AT), o-benzenediol (catechol, CH), hydroquinone (p-benzenediol, HQ), p-phenylene diamine (PD) and copper sulphate (CS, for Chamelaucium). The catechol oxidase inhibitors were tropolone (TP), 4-hexylresorcinol (HR) and 2,3-dihydroxynaphthalene (DN). A laccase inhibitor, cetyltrimethylammonium bromide (CM), was also used. 2-Mercaptoethanol (ME), phenyl hydrazine (PH) and salicylhydroxamic acid (SH) were tested as inhibitors of both peroxidase and phenoloxidase. More specific phenoloxidase inhibitors used were p-chlorophenol (CP), p-nitrocathechol (NC), p-nitrophenol (NP) and sodium metabisulphite (SM). All chemicals were AR Grade from Sigma–Aldrich, except for CS and SM from BDH Ajax Chemicals. ME and PH were supplied as liquid formulations. Some inhibitors were initially dissolved in 70% ethanol prior to being diluted with DI to 200 mL; viz. 7.5 mL ethanol for SH and 5 mL for PD, DN, HR, TP, CP and NP. Sets of control stems were maintained for the highest ethanol concentration supplied as a solution pulse. At test concentrations, some chemicals (CH, HQ, CS, TP, NC, NP, SM and SH) lowered pH to <5. At such values, stem blockage may be inhibited by low pH (Van Doorn and Cruz, 2000). Therefore, NaOH was used to adjust pH to ~6.0 using up to 8 drops 1 N NaOH for 200 mL. Conversely, PD and PH increased pH to >8 and H_3PO_4 was used to adjust pH to ~6.0 using up to 100 μL 0.5 M H_3PO_4 for 200 mL. Sets of control stems were maintained for the highest amounts of NaOH (8 drops of 1 N NaOH in 200 mL) and H_3PO_4 (100 μL 0.5 M H_3PO_4 in 200 mL) used. In the interest of practically sized experiments, one concentration only for each inhibitor was tested. Concentrations (Table 1) were chosen based on published findings for other species (Van Doorn and Cruz, 2000; Van Doorn and Vaslier, 2002; Vaslier and van Doorn, 2003; Loubaud and van Doorn, 2004; He et al., 2006). The inhibitors were applied as pulse treatments for 5 h, except for AT which was pulsed for 20 min. Pulsing was in the light at 20 °C and 60% R.H. Pulse treatments were followed by 24 h standing in DI water at 20 °C and 60% RH with a view to avoiding reaction of inhibitor chemicals with dilute chlorine compounds (see below) in the vase solution. Stem ends were not recut after either inhibitor pulsing or DI flushing and in the vase solution.

Table 1
Concentrations (mM) and abbreviations (Abb.) for oxidase enzyme inhibitors tested for *Acacia holosericea* and *Chamelaucium uncinatum*.

Inhibitors	Abb.	mM
Peroxidase inhibitors		
3-Amino-1,2,4-triazole (amitrol)	AT	2
o-Benzene diol (catechol)	CH	5
p-Benzenediol (hydroquinone)	HQ	5
p-Phenylenediamine	PD	10
Copper sulphate (for waxflower)	CS	2
Catechol oxidase inhibitors		
2,3-Dihydroxynaphthalene	DN	10
4-Hexylresorcinol (4-HR)	HR	5
2-Hydroxy-2,4,6-cycloheptatrienone (tropolone)	TP	5
Laccase inhibitor		
Cetyltrimethylammonium bromide (CTMBA)	CM	10
Phenoloxidase inhibitors		
p-Chlorophenol	CP	10
4-Nitrocatechol sulphate (dipotassium salt)	NC	5
p-Nitrophenol	NP	5
Sodium metabisulphite	SM	10
Peroxidase and phenoloxidase inhibitors		
2-Mercaptoethanol (liquid)	ME	10
Phenyl hydrazine (liquid)	PH	10
Salicylhydroxamic acid (SHA)	SH	10

2.3. Vase life evaluation

After DI flushing, stems were stood into individual plastic vases with 200 mL of DI containing 10 mg L^{-1} available chlorine (sodium salt of dichloroisocyanuric acid; DICA) as an anti-microbial (Joyce et al., 2000). Mouths of each plastic vase were covered with Parafilm™ to keep foreign matter out and prevent evaporation of solution. Stems were inserted through a slit in the plastic cover. A temperature and relative humidity data logger (Tinytag®, Hastings Data Loggers) was used for recording conditions in the vase life room operated at 21–23 °C and ~70% RH with 12 h light day^{-1} (PAR photon flux ~15 μmol m^{-2} s^{-1}; 0700–1900 h). Leaf wilting, tip

bending and other visible symptoms of deterioration were recorded daily from day 0 when stems were first placed into vases. For Acacia stems from young plants, vase life was judged terminated when almost half of the stem showed bending from the tip, when phyllodes (leaves) on the upper stem became slightly shrivelled in form due to bending/wilting, or when phyllodes on the lower stem lost turgidity and their silky and silvery appearance and softness, becoming dull in appearance due to desiccation. For stems from established plants, vase life was considered terminated when the first phyllode on the lower stem lost turgidity and started to desiccate. With Chamelaucium, vase life was terminated when half of the flowers on a sprig had closed such that their petals formed an angle of <45° to the receptacle and style (Joyce, 1993) or abscised.

2.4. Water uptake, water loss and relative fresh weight

Weighing, using an analytical balance, of vases with (A) and without cut stems (B) was commenced on day 0 and continued daily (every 2 days for Chamelaucium) during the vase life period. Water uptake (WU) and water loss (WL, transpiration) rates and relative fresh weight (FW) were calculated by the formulae: WU ($g\,g^{-1}$ initial fresh weight-FW) = $B_{n-1} - B_n$/initial FW ($A_0 - B_0$); WL ($g\,g^{-1}$ initial FW) = $A_{n-1} - A_n$/initial FW ($A_0 - B_0$); RFW (%) = $[(A_n - B_n)/(A_0 - B_0)] \times 100$; where A is the weight of vase with the cut stem (i.e. vase + solution + stem, g), B is the weight of vase without the cut stem (i.e. vase + solution, g), B_{n-1} is the weight (g) on the previous day, A_0 and B_0 are the weights (g) on day 0, and A_n and B_n are the weights (g) at day n, for $n = 1, 2, 3$, etc.

2.5. Experiment design and analysis

Experiments were arranged as randomised complete block (RCB) designs. Numbers of treatments were 19 (20 for Chamelaucium), comprised of 4 (5 for Chamelaucium) peroxidase inhibitors, 8 phenoloxidase inhibitors and 3 peroxidase and phenoloxidase inhibitors (Table 1), plus 4 controls: viz. DI, ethanol, NaOH and H_3PO_4. Replicates were five foliage stems with each inhibitor experiment for Acacia and 10 flowering stems for Chamelaucium. Matched stem samples on the basis of length, thickness, etc. were used within replicates for all treatments. Data for each day were analysed using two way ANOVA and F-test at $P \leq 0.05$ with SAS version 9.3. Treatment effects were partitioned using contrasts. The first contrast test was within controls and showed no significant variation between the four controls (data not presented). Thus, individual contrast tests were applied and are presented between each inhibitor and the averaged control. The same tests were applied between all inhibitors and all controls (data not presented). Standard errors (SEs) are presented in vase life figures.

3. Results

3.1. Relative fresh weight, water uptake and water loss

3.1.1. Experiment 1 for Acacia

Effects of enzyme inhibitor pulse treatments on changes in relative fresh weight (RFW), water uptake (WU) and water loss (WL) for stems from young Acacia plants over 8 days in the vase were recorded (Fig. 1). Significant treatment effects were observed for WL, WU and RFW (Table 2).

AT had no effects on RFW (Fig. 1A), WU (Fig. 1AU) and WL (Fig. 1AL) (Table 2). Other peroxidase inhibitors were significantly positively effective: CH, from day 4 for RFW (Fig. 1A), on day 2 for WU (Fig. 1AU) and WL (Fig. 1AL); and HQ, on day 6 for RFW (Fig. 1A); PD, from day 6 for RFW (Fig. 1A), from day 4 for WU (Fig. 1AU) and WL (Fig. 1AL) (Table 2).

All catecholoxidase and laccase inhibitors showed significant positive effects from day 4 for RFW (Fig. 1B), WU (Fig. 1BU) and WL (Fig. 1BL) (Table 2). CM for WL and RFW, HR for WU, and TP for RFW were not significantly effective on day 2 (Table 2). The other four phenoloxidase inhibitors showed variable effects for RFW (Fig. 1C), WU (Fig. 1CU) and WL (Fig. 1CL). CP was significantly positively effective compared to the control on days 4 and 6 for RFW, on day 2 for WU and WL (Fig. 1C, CU, CL). NC was significantly effective every day for RFW and WU and on day 8 for WL (Fig. 1C, CU, CL). NP was significantly positively effective from day 4 for RFW and on day 2 for WU (Fig. 1C, CU). SM showed positive effects until day 6 on RFW and on day 2 for WU and WL (Fig. 1C, CU, CL).

The three inhibitors of both peroxidase and phenoloxidase enzymes had significant positive effects for RFW (Fig. 1D), WU (Fig. 1DU) and WL (Fig. 1DL) (Table 2). ME was significantly positively different every day for RFW, on day 2 for WU and on days 2 and 8 for WL. PH had a positive effect on day 8 for RFW. For Acacia stems from young plants, SH was significantly positively effective compared to control every day for RFW and WL, and except the last day for WU.

3.1.2. Experiment 2 for Acacia

Effects of the enzyme inhibitor treatments on changes for RFW, WU and WL of cut Acacia stems from established plants over 8 days are presented in Fig. 2. Significant treatment effects in the experiment were evident for WL, WU and RFW (Table 3).

AT and CH had no effects on RFW, WU and WL (Fig. 2A, AU, AL; Table 3). Another peroxidase inhibitor, HQ, was significantly positive relative to the control from day 6 for RFW and WU, on day 8 for WL (Fig. 2A, AU, AL; Table 3). PD increased RFW from day 4 and decreased WL on day 2 (Fig. 2A, AL; Table 3).

Similar to experiment 1, catecholoxidase and laccase inhibitors showed significant positive effects for RFW, WU and WL (Fig. 2B, BU, BL; Table 3). DN maintained RFW and increased the WU on day 8, but had no significant effect on WL. HR and TP were significantly and positively different to the control for RFW, WU and WL. Positive effect of CM was significant from day 6 for RFW and WU, and on day 8 for WL. Effects of other phenoloxidase inhibitors on RFW, WU and WL are given in Fig. 2C, CU, CL and Table 3. The only significant positive effect of CP was on the last day for RFW. NC was significantly positively effective everyday for RFW and on day 6 for WU. NP had no significant effect. SM significantly increased WL, WU and RFW.

Effects of the inhibitors of both peroxidase and phenoloxidase enzymes are presented in Fig. 2D, DU, DL and Table 3. For Acacia stems from established plants, ME and SH had no significant effects. PH was positively effective for WL and WU from day 6 and always effective for RFW.

3.1.3. Experiment for Chamelaucium

Effects of pulse treatments with oxidative enzyme inhibitors on changes in RFW, WU and WL for Chamelaucium over 8 days are shown in Fig. 3, with the significance of effects being shown in Table 4.

Most peroxidase (Fig. 3A, AU, AL), catecholoxidase and laccase (Fig. 3B, BU, BL), other phenoloxidase (Fig. 3C, CU, CL), and peroxidase and phenoloxidase (Fig. 3D, DU, DL) inhibitors often had significant positive effects compared to the control for RFW, WU and WL (Table 4).

However, AT, HQ, ME and SH had no significant effects on water relations, except SH for WL on day 6 (Table 4). CH was significantly and positively different to the control for WL and WU on days 4 and 6 and for RFW on days 2 and 4. PD and PH had significant positive effects for WL and WU from day 4, and every day for RFW. CS had mostly significant positive effects, except on day 4 for WL and WU and on day 2 for RFW.

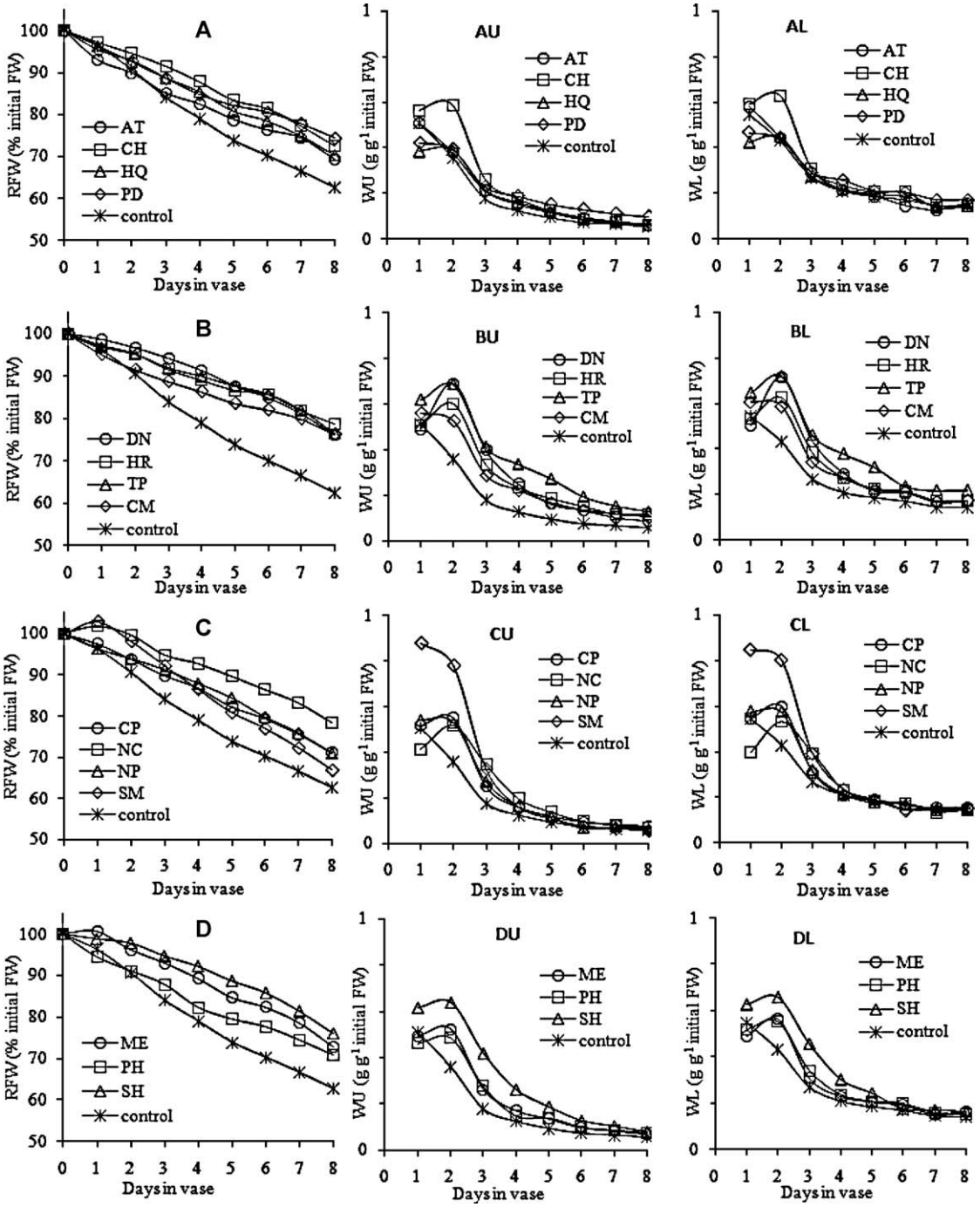

Fig. 1. Effects of pulsing with oxidase enzyme inhibitors on changes in relative fresh weight (RFW; A, B, C, D), water uptake (WU; AU, BU, CU, DU) and water loss (WL; AL, BL, CL, DL) for *Acacia holosericea* over 8 days in the vase in experiment 1 with stems from young (1-year-old) plants. Data are means of 5 replicates. A, AU, AL: peroxidase inhibitors – amitrol (AT), catechol (CH), hydroquinone (HQ) and p-phenylenediamine (PD). B, BU, BL: catechol oxidase inhibitors – 2,3-dihydroxy naphthalene (DN), 4-hexylresorcinol (HR) and tropolone (TP); and laccase inhibitor cetyltrimethylammonium bromide (CM). C, CU, CL: phenoloxidase inhibitors – p-chlorophenol (CP), 4-nitrocatechol sulphate (NC), p-nitrophenol (NP) and sodium metabisulphite (SM). D, DU, DL: peroxidase and phenoloxidase inhibitors – 2-mercaptoethanol (ME), phenyl hydrazine (PH) and salicylhydroxamic acid (SH).

DN, NC, NP and TP were phytotoxic at the concentrations applied. Significant effects of DN, NC, HR and SM were negative for WL, WU and RFW (Fig. 3; Table 4). The significant effect of NP for RFW on day 8 was also negative.

CM had significant positive effects from day 4 for WL and WU and on days 2 and 4 for RFW (Fig. 3; Table 4). CP was significantly positively effective for WU on days 4 and 6, and for WL and RFW on days 2–6. For Chamelaucium, PH had significant positive effects from day 4 for WL and WU and every day for RFW.

3.2. Vase life

Effects of peroxidase and phenoloxidase enzyme inhibitors on vase life of Acacia in two experiments and Chamelaucium in one experiment are shown in Table 5. Compared to the control, AT had no effect for Acacia, but significantly increased the vase life for Chamelaucium. CH was significantly positively effective for both species. HQ had no effect for Chamelaucium, but significantly increased the vase life for Acacia in experiments 1 and 2. The

Table 2
Significance of effects (*P*-values for $P > 0.05$ or $^{*}P \leq 0.05$ and $^{**}P \leq 0.01$ from ANOVA) for pulse treatments with peroxidase (perox.) and phenoloxidase (phenolox.) enzyme inhibitors (E.I.) on water loss (WL), water uptake (WU) and relative fresh weights (RFW) of *Acacia holosericea* on days 2, 4, 6 and 8 in the vase in experiment 1 with stems from young plants.

	Day	E.I.														
		Peroxidase				Catecholoxidase–laccase				Phenoloxidase				Perox. + phenolox.		
		AT	CH	HQ	PD	DN	HR	TP	CM	CP	NC	NP	SM	ME	PH	SH
WL	2	0.97	*	0.86	0.97	**	*	**	0.07	*	0.13	0.08	**	*	0.18	**
	4	0.87	0.12	0.45	*	**	**	**	**	0.62	0.10	0.42	0.10	0.17	0.25	**
	6	0.89	0.73	0.52	**	**	**	**	**	0.44	0.13	0.78	0.65	0.36	0.42	**
	8	0.14	0.18	0.23	**	**	**	**	**	0.07	*	0.11	0.14	**	0.11	**
WU	2	0.96	*	0.72	0.53	**	0.06	**	*	*	*	*	**	*	0.11	**
	4	0.53	0.14	0.24	*	**	**	**	**	0.51	*	0.39	0.92	0.23	0.60	**
	6	0.53	0.56	0.11	**	**	**	**	**	0.28	*	0.53	0.40	0.22	0.22	**
	8	0.89	0.86	0.32	**	**	**	**	**	0.12	*	0.95	0.36	0.16	0.46	0.08
RFW	2	0.65	0.08	0.44	0.35	**	*	0.07	0.72	0.22	**	0.14	**	*	0.88	**
	4	0.44	*	0.07	0.10	**	**	**	*	*	**	**	*	**	0.36	**
	6	0.21	**	*	*	**	**	**	**	*	**	*	0.09	**	0.07	**
	8	0.19	*	0.07	**	**	**	**	**	0.08	**	*	0.30	*	*	**

effect of PD in the first experiment on Acacia was not significant, but was significant in the second experiment, and was also significant for Chamelaucium. CS was tested only for waxflower and was significantly and positively effective. All three catechol oxidase inhibitors showed significant positive effects on vase life for Acacia in experiment 2. However, only HR was significantly effective in experiment 1. HR was not effective for Chamelaucium and the other two inhibitors of catechol oxidase, DN and TP, were phytotoxic, significantly reducing vase life. The laccase inhibitor CM significantly increased the vase life for Acacia in both experiments, but not for Chamelaucium. Other phenoloxidase inhibitors had significant positive effects on vase life for Acacia in experiment 1, except SM. However, SM markedly increased the vase life of Acacia compared to the control in experiment 2. NC was another effective inhibitor in both experiments with Acacia. Effects of CP and NP were not significant in experiment 2 with Acacia. CP and SM had no significant effect on vase life for Chamelaucium and NC and NP significantly reduced its vase life.

The three inhibitors of both peroxidase and phenoloxidase enzymes had mixed effects for Acacia in both experiments and Chamelaucium. The significant positive inhibitors effects were SH in experiment 1 and PH in experiment 2 for Acacia, and ME and PH for Chamelaucium.

There were highly significant correlations between vase life and WU and vase life and RFW. In Acacia experiment 1 vase life was correlated with RFW on days 2–8 with $P \leq 0.01$ and $r = 0.42$–0.70 (range for different days). Vase life was correlated with WU on days 2–5 with $P \leq 0.01$ and $r = 0.32$–0.55. In Acacia experiment 2, vase life was correlated with RFW on days 1–8 with $P \leq 0.01$ and $r = 0.50$–0.82. Vase life was correlated with WU on days 1, 2, 7 and 8 with $P \leq 0.05$ and $r = 0.23$–0.40. In Chamelaucium, vase life was correlated with RFW on days 4, 6 and 8 with $P \leq 0.01$ and $r = 0.36$–0.75. Vase life was correlated with WU on days 4, 6 and 8 with $P \leq 0.01$ and $r = 0.39$–0.62. However, the inhibitor treatments that increased WU throughout an experiment increased RFW only 54% and vase life only 36% of the time. Treatments that increased RFW throughout an experiment increased vase life 50% of the time. These calculations excluded treatments that caused visible toxicity to Chamelaucium.

4. Discussion

Peroxidase inhibitors showed positive effects for WL, WU and RFW of Acacia stems in experiments 1 and 2. PD increased WU and RFW in 56% of measurements (Tables 2 and 3). Except for AT, the other three peroxidase inhibitors, CH, HQ and PD, prolonged vase life Acacia stems in both experiments (Table 5). PD and CS increased WU and RFW of Chamelaucium (Table 4). Three peroxidase inhibitors also increased Chamelaucium vase life (Table 5). That HQ was ineffective is possibly because the concentration used

Table 3
Significance of effects (*P*-values for $P > 0.05$ or $^{*}P \leq 0.05$ and $^{**}P \leq 0.01$ from ANOVA) for pulse treatments with peroxidase (perox.) and phenoloxidase (phenolox.) enzyme inhibitors (E.I.) on water loss (WL), water uptake (WU) and relative fresh weights (RFW) of *Acacia holosericea* on days 2, 4, 6 and 8 in the vase in experiment 2 with stems from established plants. The significant effect of PD on WL was negative.

	Day	E.I.														
		Peroxidase				Catecholoxidase–laccase				Phenoloxidase				Perox. + phenolox.		
		AT	CH	HQ	PD	DN	HR	TP	CM	CP	NC	NP	SM	ME	PH	SH
WL	2	0.50	0.25	0.24	*	0.86	**	**	0.64	0.52	0.58	0.62	**	0.48	0.84	0.51
	4	0.63	0.31	0.66	0.06	0.83	0.07	*	0.62	0.66	0.31	0.38	**	0.71	0.57	0.83
	6	0.70	0.80	0.26	0.68	0.14	**	*	0.12	0.84	0.09	0.10	**	0.92	**	0.75
	8	0.38	0.67	*	0.18	0.08	**	**	**	0.74	0.09	0.76	**	0.74	**	0.48
WU	2	0.37	0.14	0.14	0.15	0.62	**	**	0.33	0.73	0.19	0.48	**	0.51	0.84	0.74
	4	0.61	0.40	0.29	0.17	0.77	**	**	0.27	0.57	0.07	0.16	**	0.92	0.25	0.72
	6	0.52	0.84	*	0.76	0.08	**	**	**	0.81	*	0.22	**	0.84	**	0.94
	8	0.28	0.85	**	*	*	**	**	**	0.37	0.08	0.82	**	0.33	**	0.55
RFW	2	0.68	0.24	0.67	0.08	*	**	*	0.13	0.27	**	0.56	**	0.50	**	0.10
	4	0.71	0.16	0.14	*	0.08	**	*	0.07	0.25	**	0.27	**	0.90	**	0.18
	6	0.74	0.15	*	**	*	**	**	**	0.20	**	0.39	**	0.59	**	0.27
	8	0.53	0.10	**	**	*	**	**	**	*	**	0.71	**	0.19	**	0.26

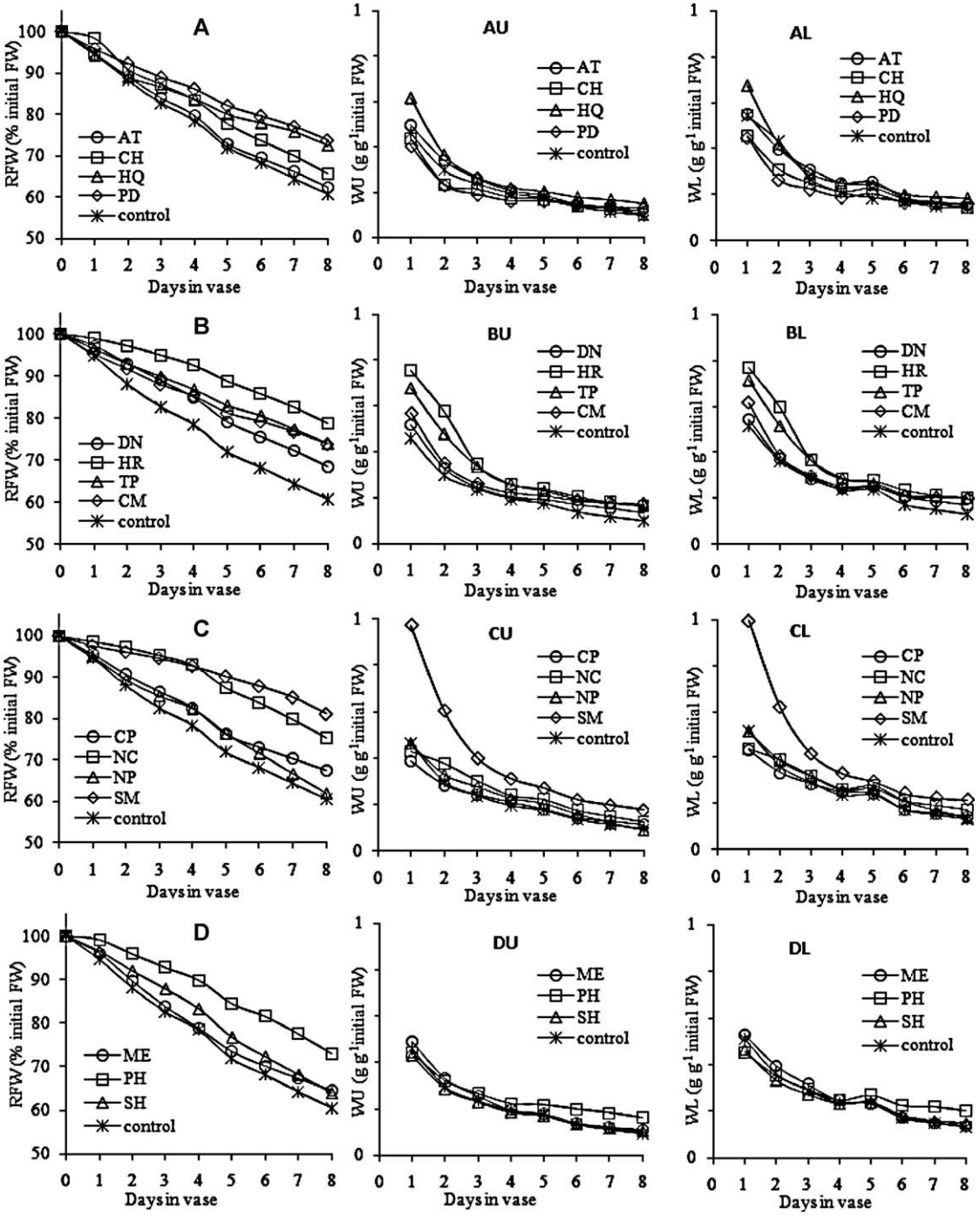

Fig. 2. Effects of pulsing with oxidase enzyme inhibitors on changes in relative fresh weight (RFW; A, B, C, D), water uptake (WU; AU, BU, CU, DU) and water loss (WL; AL, BL, CL, DL) for *Acacia holosericea* over 8 days in the vase in experiment 2 with stems from established (3–4-year-old) plants. Data are means of 5 replicates. A, AU, AL: peroxidase inhibitors – amitrol (AT), catechol (CH), hydroquinone (HQ) and p-phenylenediamine (PD). B, BU, BL: catechol oxidase inhibitors – 2,3-dihydroxy naphthalene (DN), 4-hexylresorcinol (HR) and tropolone (TP); and laccase inhibitor cetyltrimethylammonium bromide (CM). C, CU, CL: phenoloxidase inhibitors – p-chlorophenol (CP), 4-nitrocatechol sulphate (NC), p-nitrophenol (NP) and sodium metabisulphite (SM). D, DU, DL: peroxidase and phenoloxidase inhibitors – 2-mercaptoethanol (ME), phenyl hydrazine (PH) and salicylhydroxamic acid (SH).

was sub-optimum for this species. Considered collectively, these results with a broad range of inhibitors suggest a role of peroxidase enzymes in Acacia and Chamelaucium cut stem wound reactions.

The three catechol oxidase inhibitors used in this study, DN, HR and TP, generally increased WL, WU, RFW and increased vase life for Acacia foliage in 67% of measurements (Tables 2, 3 and 5). The phenoloxidase laccase inhibitor CM also improved WL, WU and RFW for both species (Tables 2–4) and vase life for Acacia in both experiments, although it had no effect on vase life for Chamelaucium (Table 5). As for peroxidase inhibitors, the generally positive effects of all catechol oxidase and laccase inhibitors suggest a role for phenoloxidase enzymes in deteriorating water relations of cut Acacia stems. The catechol oxidase and laccase inhibitors have positive effects more often than the other inhibitors, which is similar to the results of Van Doorn and Vaslier (2002).

Like catechol oxidase–laccase inhibitors, other phenoloxidase inhibitors increased WU, RFW and vase life of Acacia approximately half of the time (Tables 2 and 3). In particular, NC and

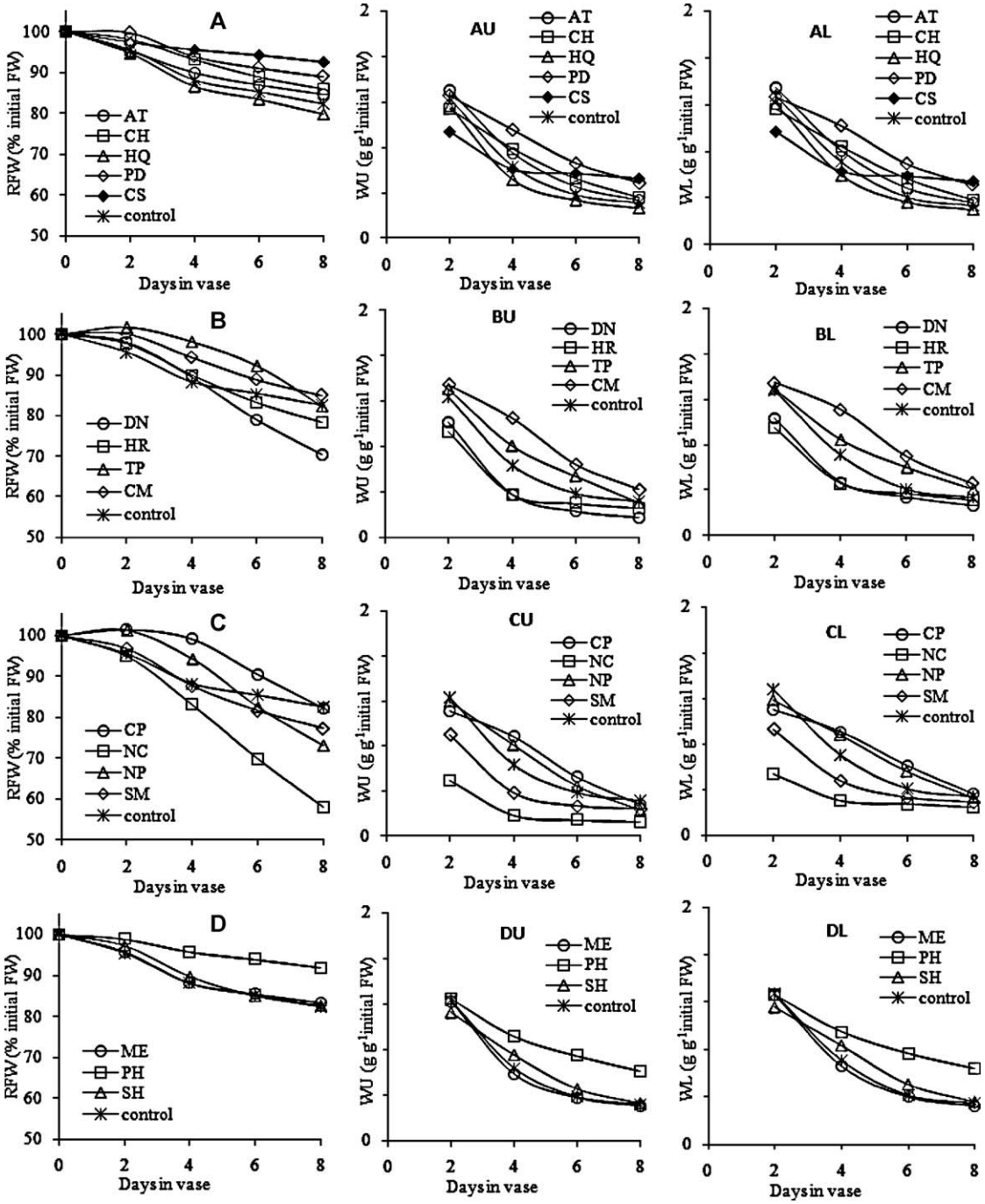

Fig. 3. Effects of pulsing with oxidase enzyme inhibitors on changes in relative fresh weight (RFW; A, B, C, D), water uptake (WU; AU, BU, CU, DU) and water loss (WL; AL, BL, CL, DL) of *Chamelaucium uncinatum* over 8 days in the vase. Data are means of 10 replicates. A, AU, AL: peroxidase inhibitors – amitrol (AT), catechol (CH), hydroquinone (HQ), p-phenylenediamine (PD) and copper sulphate (CS). B, BU, BL: catechol oxidase inhibitors – 2,3-dihydroxy naphthalene (DN), 4-hexylresorcinol (HR) and tropolone (TP); and laccase inhibitor cetyltrimethylammonium bromide (CM). C, CU, CL: phenoloxidase inhibitors – p-chlorophenol (CP), 4-nitrocatechol sulphate (NC), p-nitrophenol (NP) and sodium metabisulphite (SM). D, DU, DL: Peroxidase and phenoloxidase inhibitors – 2-mercaptoethanol (ME), phenyl hydrazine (PH) and salicylhydroxamic acid (SH).

SM consistently increased WU and RFW. This finding suggests a role of phenoloxidases in physiological plugging of stems for both woody stemmed species. That DN, TP, NC and NP were phytotoxic to Chamelaucium is indicative of concentrations being too high for this particular species (Table 4).

The inhibitors ME, PH and SH, which inhibit both peroxidase and phenoloxidase enzymes, increased WU, RFW and vase life in approximately half of the measurements. SH in Acacia experiment 1 and PH in Acacia experiment 2 and Chamelaucium increased WU and RFW in almost all measurements. These results support a contributory role of these enzymes in restricting vase water uptake (Tables 2–4).

Overall, four (five for Chamelaucium) peroxidase inhibitors, eight phenoloxidase inhibitors and three peroxidase and phenoloxidase inhibitors were tested in each experiment. In more than 50% of measurements, these increased WU, RFW and vase life. These findings indicate that both peroxidase and phenoloxidase enzymes are involved in wound-induced xylem occlusion. Moreover, similar

Table 4
Significance of effects (*P*-values for $P > 0.05$ or $^{*}P \leq 0.05$ and $^{**}P \leq 0.01$ from ANOVA) for pulse treatments with enzymes inhibitors for water loss (WL), water uptake (WU) and relative fresh weight (RFW) of *Chamelaucium uncinatum* on days 2, 4, 6 and 8 in the vase. DN, TP, NC and NP were phytotoxic (^{tx}) at the applied concentrations. Significant effects of DN, HR, NC and SM were negative and effect of NP for RFW on day 8 was also negative.

	Day	E.I.															
		Peroxidase					Catecholoxidase–Laccase				Phenoloxidase				Perox. + Phenolox.		
		AT	CH	HQ	PD	CS	DN^{tx}	HR	TP^{tx}	CM	CP	NC^{tx}	NP^{tx}	SM	ME	PH	SH
WL	2	0.32	0.11	0.35	0.84	**	**	**	0.96	0.34	**	**	0.22	**	0.82	0.79	0.09
	4	0.16	*	0.09	**	0.22	**	**	0.07	**	**	**	**	**	0.49	**	0.07
	6	0.14	**	0.33	**	**	0.11	0.40	**	**	**	**	**	0.09	0.83	**	*
	8	0.63	0.17	0.23	**	**	**	0.39	0.06	**	0.53	**	0.75	0.10	0.60	**	0.65
WU	2	0.34	0.21	0.29	0.71	**	**	**	0.39	0.12	0.09	**	0.65	**	0.85	0.85	0.15
	4	0.10	*	0.08	**	0.68	**	**	*	**	**	**	**	**	0.46	**	0.09
	6	0.17	**	0.31	**	**	**	0.10	**	**	**	**	0.19	*	0.85	**	0.14
	8	0.56	0.21	0.16	**	**	**	0.16	0.83	**	0.35	**	*	*	0.76	**	0.59
RFW	2	0.74	*	0.42	**	0.14	*	0.08	**	**	**	0.61	**	0.28	0.82	**	0.14
	4	0.23	**	0.25	**	**	0.41	0.22	**	**	**	**	**	0.70	0.93	**	0.30
	6	0.39	0.07	0.30	**	**	**	0.25	**	0.06	**	**	0.12	*	0.96	**	0.82
	8	0.30	0.12	0.20	**	**	**	0.06	0.94	0.23	0.92	**	**	*	0.67	**	0.95

effects of oxidase inhibitors are previously reported for Chrysanthemum (Van Doorn and Vaslier, 2002; Vaslier and van Doorn, 2003), Astilbe (Loubaud and van Doorn, 2004) and Grevillea (He et al., 2006).

van Meeteren and Arévalo-Galarza (2009) suggested a major role of PAL in xylem occlusion caused by wounding of Chrysanthemum stems. Further evidence for involvement of PAL comes from observations that S-carvone, an inhibitor of PAL activity, increased water uptake and vase life of *B. frutescens*, *C. uncinatum* and Grevillea, but not of *A. holosericea* (He et al., 2006; Damunupola et al., 2010). The inhibitors used in the present study were unlikely to inhibit PAL activity, since the reaction that this enzyme catalyses is not oxidation.

WL (transpiration) data were presented herein to separate postulated occlusion of vase water uptake effects from possible anti-transpiration effects. The various oxidative enzyme inhibitors had generally similar effects in that they increased WL, WU and RFW in concert. More than half of the inhibitor treatments increased all three water relation parameters. In only one instance did an inhibitor decrease WL and increase RFW; viz. PD inhibited WL on day 2 for Acacia in experiment 2 (Table 3). Thus, oxidative enzyme inhibitors typically did not inhibit transpiration and, accordingly, their effects were likely directed against occlusion.

It was also possible that inhibitors could have acted by increasing WL directly, with WU increasing as a consequence. However, only one report was found in the literature where an inhibitor increased transpiration without causing phytotoxicity. In this case, CS at <1 μM was supplied through the roots in a hydroponic solution for Chinese mustard plants (*Brassica juncea* L.; Li et al., 2009). While the possibility that some inhibitors may have increased transpiration (WL) and hence uptake cannot be ruled out, the fact that over half of the oxidative enzymes inhibitors increased uptake supports the hypothesis that these compounds increased WU by inhibiting the activity of these enzymes.

Vase life was significantly correlated with the WU measured on 50–75% of days in the three experiments. However, only approximately 36% of treatments that increased WU also increased vase life. This is possibly because vase life can be mediated by other physicochemical processes pertaining to water balance in particular (e.g. turgor pressure, transpiration and desiccation) and senescence in general (e.g. oxidative stress and ethylene perception) (Halevy and Mayak, 1981). In addition, the various inhibitors may have clinical or sub-clinical toxic effects on vase life (Table 5; He et al., 2006). Also, the oxidative enzyme inhibitor compounds used in the current work may have alternative effects. For example, CM and CS can act as biocides and CM also as a surfactant (Damunupola and Joyce, 2008). In this context, Loubaud and van Doorn (2004) noted caution in the course of reporting their work with inhibitors as a pulse treatment before dry storage. In the present work, early bacterial development seemed unlikely as stems were rinsed and recut under DI water immediately before placing them into the inhibitor pulse treatment solutions. Moreover, the pulse treatment period was generally only 5 h and after pulsing the stems were flushed with DI water for 24 h before being placed individually into vases containing DICA biocide. Had inhibitors been supplied continuously in the vase water, then any biocidal effect could more likely confound any inhibitory effect on oxidative enzymes.

Physiological stem blockage was previously proposed for a number of relatively more herbaceous [e.g. Chrysanthemum (Van Doorn and Cruz, 2000; Van Doorn and Vaslier, 2002) and Bouvardia (Van Doorn and Cruz, 2000; Van Doorn and Vaslier, 2002; Vaslier and

Table 5
Effects of pulse treatments with peroxidase and phenoloxidase enzyme inhibitors (E.I.) on vase life of *Acacia holosericea* and *Chamelaucium uncinatum*. Data are means ± S.E. of five replications for *Acacia* experiments and 10 replicates for *Chamelaucium*.

Inhibitors	*Acacia* exp. 1	*Acacia* exp. 2	*Chamelaucium*
Peroxidase E.I.			
Amitrol (AT)	7.0 ± 0.5 ns	6.2 ± 0.4 ns	14.9 ± 0.5**
Catechol (CH)	8.4 ± 1.2*	7.4 ± 0.8*	15.2 ± 0.7**
Hydroquinone (HQ)	8.2 ± 0.2*	7.8 ± 0.7**	11.7 ± 1.0 ns
p-Phenylenediamine (PD)	8.0 ± 0.0 ns	8.0 ± 0.3**	15.5 ± 0.7**
Catecholoxidase–laccase E.I.			
Copper sulphate (CS)	–	–	16.2 ± 0.6**
2,3-Dihydroxynaphthalene (DN)	8.0 ± 0.9 ns	7.2 ± 0.6*	8.9 ± 0.3 tx
4-Hexylresorcinol (HR)	8.4 ± 0.2*	8.4 ± 0.2**	11.1 ± 0.7 ns
Tropolone (TP)	8.0 ± 0.0 ns	7.8 ± 0.5**	8.3 ± 0.4 tx
CTMBA (CM)	8.8 ± 0.6**	8.8 ± 0.2**	12.1 ± 0.8 ns
Phenoloxidase E.I.			
p-Chlorophenol (CP)	8.5 ± 0.8*	6.8 ± 0.9 ns	10.9 ± 0.5 ns
4-Nitrocatechol sulphate (NC)	9.4 ± 0.7**	7.2 ± 0.6*	6.1 ± 0.1 tx
p-Nitrophenol (NP)	8.2 ± 0.2*	6.2 ± 0.5 ns	8.9 ± 0.5 tx
Sodium metabisulphite (SM)	8.0 ± 0.0 ns	11.0 ± 0.6**	11.6 ± 0.8 ns
Perox. + phenolox. E.I.			
2-Mercaptoethanol (ME)	7.4 ± 0.6 ns	6.4 ± 0.7 ns	13.6 ± 0.8**
Phenyl hydrazine (PH)	8.0 ± 0.0 ns	7.6 ± 0.7*	16.2 ± 0.8**
Salicylhydroxamic acid (SH)	8.8 ± 0.6**	7.0 ± 0.6 ns	12.7 ± 0.8 ns
Control	7.0 ± 0.4	6.0 ± 0.4	11.4 ± 0.6

ns: not significant, tx: phytotoxic.
* Significant at $P \leq 0.05$.
** Significant at $P \leq 0.01$.

van Doorn, 2003; Loubaud and van Doorn, 2004)] and also comparatively woody [e.g. Grevillea (He et al., 2006); *H. francisiana* (Williamson et al., 2002) and *Syringa vulgaris* (Van Doorn et al., 1991; Jedrzejuk and Zakrzewski, 2009)] species. In contrast, no evidence was found of physiological blockage for cut Rose and Viburnum flowers. Rather occlusion was determined to be bacterial in origin or nature (Loubaud and van Doorn, 2004). However, in the present study with very woody Acacia and Chamelaucium stems, physiological occlusion, presumably of the xylem, was strongly inferred with the broad range of oxidase inhibitors applied at concentrations already shown to be effective with other cut flower and foliage species.

While wound reactions might be anticipated for all cut woody stems, the degree of response may logically be modulated by both internal plant and external climate and management variables (Halevy and Mayak, 1981). Plant variables would be genotypic as well as developmental, e.g. maturity. In the present study and while not directly compared in a single experiment, Acacia stems from young versus mature plants growing nearby responded somewhat differently to some inhibitors. In particular, effects of SM, PH, ME and SH differed across the two Acacia experiments. Morphologically, phyllodes on stems from the different-aged source plants were observed to vary in size and shape (data not presented) and in characteristic cut foliage wilting symptoms (see Section 2).

The findings that inhibitors of wound-induced enzymes extended the vase life of cut *A. holosericea* and *C. uncinatum* appears to be potentially useful in a practical context. However, most of the inhibitors used are considered highly (AT, HQ, PD, CM, ME, PH and SM) or moderately (CH, CS, HR, CP and NP) hazardous chemicals, although some (DN, TP, SH and NC) are a low hazard ('Chemwatch' Material Safety Data Sheets). Moreover, most are relatively costly chemicals which could limit any commercial use. Thus, most of the oxidative enzyme inhibitors are likely too expensive and/or too toxic to be practically used. Nonetheless, among the broad range of oxidase inhibitors tested, CS and SM are relatively inexpensive and not overly harmful, and so may merit consideration in an applied treatment context.

In conclusion, indirect evidence obtained with a range of oxidative enzyme inhibitors is presented herein for physiological occlusion of water uptake in Acacia cut foliage and Chamelaucium cut flower stems. Wound-induced enzyme activities are generally considered to contribute to phenolic and possibly suberin deposition (see Section 1). Phenolic metabolism in response to wounding is likely associated with ethylene signalling (Heredia and Cisneros-Zevallos, 2009). On this basis, further studies into harvest wound-associated physiological blockage of the vasculature are warranted for these two woody stemmed floriculture species. This future research could include correlating measured oxidative enzymes activity with water conductivity in their stems.

Acknowledgements

Prof. Fisun Çelikel was the grateful recipient of an Endeavour Research Fellowship Award from the Australian Government. Mayati Che Husin (PhD student) and Dr. Iftikhar Ahmad Hashmi (visiting researcher) are thanked for their technical assistance in some experiments. Allan Lisle is gratefully recognised for advice on experimental design and assistance with statistical analyses. We thank the reviewers for valuable comments.

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