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(S)-(-)-limonene fumigation protects waxflowers (*Chamelaucium spp.*) from detrimental effects of ethylene on abscission of flowers/buds

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Abstract

Waxflowers are economically important native cut flowers in Australian floriculture industry. Postharvest flowers/buds abscission on stems of waxflowers causes serious economic losses to the Australian waxflower industry. The effects of (*S*)-(-)-limonene in reducing the damaging effects of ethylene on abscission of flowers/buds in six varieties of Geraldton waxflowers 'WX73', 'WXFU' ,'WX17', 'WX58', 'WX56' and 'Purple Pride' were investigated in 2014 and 2015. All the experiments were conducted having four treatments. The flowers stems were fumigated with ethylene (10 μLL^{-1}) for 24 h and (*S*)-(-)-limonene (1 µM) alone for 18 h and (*S*)-(-)-limonene fumigation followed by exposure to ethylene. Untreated sprigs served as control. All the six experiments were laid out by following two-factor factorial completely randomised design including the (4 treatments and 4 times) with three replications and three stems were treated as an experimental unit. Cumulative abscission of flowers/buds was calculated for four consecutive days following 24 h of ethylene exposure. Fumigation with (*S*)-(-)-limonene (1 µM) for 18 h followed by exposure to 10 μLL^{-1} ethylene significantly reduced flowers/buds abscission as compared to ethylene treatment alone in all varieties except 'WX17'. Mean flowers/buds abscission was significantly reduced when flower stems were fumigated with (*S*)-(-)-limonene followed by exposure to 10 μLL^{-1} ethylene (26.7%, 30.9%, 62.4%, 13.6 and 6.4%) as compared to those exposed to ethylene treatment alone (89.9%, 82.0%, 77.4%, 85.9% and 68.8%) in 'WX73', 'WXFU', 'Purple Pride', 'WX56' and 'WX58' respectively. 'WX17' waxflower fumigated with (*S*)-(-)-limonene followed by exposure to 10 μLL^{-1} ethylene did not show a significant reduction in flowers/buds abscission as compared to ethylene alone probably this genotype is more sensitive to ethylene because of their inherited trait. The results suggest that (*S*)-(-)-limonene act as an antagonist to ethylene action and consequently reduced flowers/buds abscission in waxflowers.

Keywords: Geraldton waxflower (*Chamelaucium uncinatum*); ethylene; abscission of flowers/buds; (S)-(-)-limonene.
Abbreviation: ABA _ abscisic acid; ANOVA_ Analysis of variance; (*S*)-(-)-Lim_ (*S*)-(-)-limonene alone; (*S*)-(−)-Lim + E _(*S*)-(-)-limonene followed by ethylene; LSD_ Least significant difference; KCl_ potassium chloride; STS_ silver thiosulphate.

Introduction

Geraldton waxflower (*Chamelaucium uncinatum* Schauer), other *Chamelaucium* species and hybrids have become one of the attractive native plants and valuable cut flowers. *Chamelaucium* species are the major cut flowers exported from Australia in the recent past due to their relatively small pretty flowers and leaves (Beasley and Joyce, 2002; Growns, 2004; Seaton et al., 2007; Vitner et al., 2007; Gollnow and Worrall, 2010; Seaton and Poulish, 2010). Waxflower stems are strong, straight and bear green leaves and attractive shiny small flowers (Yan, 2001; Dinh et al., 2008, 2011; Gollnow and Worrall, 2010). The Geraldton waxflower possesses a distinct pleasant aroma. It starts to produce nectar at anthesis that continues for 7-10 days (Olley et al., 1996; Beasley and Joyce, 2002). Plummer et al. (2001) pointed out that the colours of petals range from white through pinks to mauve and purple, with the deep pinks and purple colours the most acceptable commercially. Dinh et al. (2011) estimated that the world production of waxflowers exceeds 300 million cut stems per year. Cut waxflowers are exported from Australia to European markets and are now ranked in the top 20 of sold volume of flowers (Yan, 2001; Gollnow and Worrall, 2010; Seaton and Poulish, 2010). Extensive losses of floral organs (flowers/buds) in waxflower cut stems during transport, handling, storage and marketing can occur mainly as a result of unfavourable exposure to exogenous ethylene (Joyce, 1988; Faragher, 1989; Joyce, 1993). Faragher et al. (2010) stated that the abscission of floral organs of native Australian flowers such as *Boronia heterophylla, Backhousia myrtifolia, Baeckea virgata, Ceratopetalum gummiferum, Chamelaucium uncinatum,* some *Leptospermum, some Grevillea species, Telopea speciosissima, Thryptomene*

calycina, and Verticordia nitens, V. cooloomia, V. grandis and *V. serrata*, is caused by the presence of ethylene around the flowers. As a prelude, ethylene not only causes abscission of flowers/buds in the postharvest phase of waxflowers but also damage both flower petals and leaves consequently reducing the value of the stems and resulting in a low price in export markets (Joyce, 1993). Several methods have been tested with different cultivars of waxflowers to downregulate ethylene production and ethylene action to overcome the adverse effects of ethylene on prolonging vase life. Joyce and Jones (1992) suggested that the vase water containing 10 mg L^{-1} abscisic acid (ABA) alone or in combination with 10 mmol potassium chloride (KCl) was beneficial in promoting longevity of vase life of 'Purple Pride' and 'Alba' cultivars of waxflower. Later on, Joyce et al. (1996) stated that addition of triadimenol fungicide (10 mg L^{-1}) into vase solution with accumulation of (ABA) regulated stomatal closure and improved water balance in waxflowers stems consequently extended vase life of flowers and leaves in 'Alba', 'Mullering Brook' and 'Purple Pride'. Damunupola et al. (2010) stated that the vase solutions containing an antibacterial compound such as (*S)*-carvone (0.318 to 0.636 mM) improved foliage and flower vase life in 'Mullering Brook' Geraldton waxflower. 'Mullering Brook', 'Alba' and 'Elegance' waxflowers treated with 0.5 mmol of silver thiosulphate (STS) for 15 - 22 h at 0°C resulted in inhibition of endogenous ethylene biosynthesis consequently reducing flower abscission (Joyce,1993). Beneficial effects of 1-MCP in protecting cut flowers from ethylene action have also been reported in 'Wendy' Geraldton waxflower, (Serek et al., 1995b), *Hibiscus rosa* (Reid et al., 2002), Zonal Geraniums (*Pelargonium x hortorum*) (Jones et al., 2001), *Rosa hybrida* (Liao et al., 2013), *Dianthus caryophyllus* and *Delphinium* (Ichimura et al. 2002). Moreover, a single application of 1-MCP (10 nLL^{-1}) for 12 h during the postharvest has been reported to be the most effective method for protecting and reducing waxflower losses (Macnish et al., 2000b; Gollnow and Worrall, 2010; Seaton and Poulish, 2010).

Application of different ethylene antagonists such as STS and 1-MCP are known to reduce damaging effects of ethylene in horticultural crops (Kader, 2003). Limonene is a natural monoterpene found in citrus and other fruit and is considered as environmentally friendly, when used as an adjuvant for agricultural chemicals as registered with the Environmental Protection Agency (Ibrahim et al., 2001, Hollingsworth, 2005). Previously, insecticidal, repellent and antimicrobial activity of limonene and its potential use in controlling insect pests as spray or dipping method for harvested commodities such as vegetables, fruits or cut and potted flowers have been reported by Ibrahim et al. (2001) and Hollingsworth (2005). In addition, limonene has been classified by the U.S. Food and Drug Administration as a Generally Recognised As Safe (GRAS) compound and can be used as an additive to food or flavouring (EPA, 1994). Hollingsworth (2005) also stated that (1%) of limonene solution has no phytotoxic effects on thick and waxy leaves of ornamental plants such as orchids, palms and cycads.

In addition, some preliminary research on anti-ethylene properties of various monoterpenes in plants suggested that natural monoterpenes, like limonene compete with ethylene for the ethylene receptor but, the mode of action is yet unclear (Grichko et al., 2003). Currently, no information is available on the effect of (*S*)-(-)-limonene on inhibiting flowers/buds abscission in native waxflower or any other plant. It was hypothesised that an antagonistic effect of (*S*)-(-)-limonene on ethylene may regulate the postharvest abscission of flowers/buds in 'Geraldton wax'. Therefore, ethylene antagonistic effects of (*S*)-(-)-limonene were investigated in regulating abscission of flowers/buds on the stems of different varieties of waxflowers by exposing to (*S*)-(-)-limonene and ethylene alone and (*S*)-(-)-limonene fumigation followed by ethylene exposure.

Results

Effects of (S)-(-)-limonene fumigation followed by ethylene exposure on mean flowers/buds abscission in different varieties of waxflower

In 2014 when averaged over four-day periods, mean abscission of flowers/buds was significantly ($P \leq 0.05$) higher (89.9 % and 82.1%) for 'WX73' and 'WXFU' waxflower stems when exposed for 24 h to exogenous ethylene (10 μLL^{-1}) alone as compared to the untreated control flowers (5.5% and 9.9%) and all other treatments respectively in 2014 (Fig. 1). In addition, 'WX17' genotype mean abscission of flowers/buds was significantly ($P \leq 0.05$) increased (99.6% and 97.8%) when 'WX17' stems were fumigated with (*S*)-(-)-limonene (1 µM) followed by ethylene (10 μLL^{-1}) and also in ethylene treatment alone (Fig. 1). Meanwhile, the flower sprigs of 'WX73' and 'WXFU' exhibited significantly lower mean abscission of flowers/buds when treated with (*S*)-(-)-limonene (1 µM) for 18 h followed by exposure to 10 μLL^{-1} ethylene (26.7% and 30.9%) as compared to ethylene treatment alone (89.9 % and 82.1%) respectively (Fig. 1). The protective effects of (*S*)-(-)-limonene on flower abscission was not observed in 'WX17' in 2014 (Fig. 1). Flower stems of 'Purple Pride', 'WX56' and 'WX58' exposed to ethylene (10 μLL^{-1}) alone showed significantly ($P \leq 0.05$) increased mean abscission of flowers/buds (77.4%, 85.9% and 68.8% respectively) as compared with the untreated control flowers (43.4%, 7.10% and 1.30%) and all other treatments in 2015 (Fig. 2). Meanwhile, (*S*)-(-)-limonene (1 µM) fumigation for 18 h followed by exposure to ethylene (10 μLL^{-1}) significantly ($P \leq 0.05$) reduced mean abscission of flowers/buds in 'Purple Pride', 'WX56' and 'WX58' (62.4%, 13.6% and 6.3%) respectively (Fig. 2).

Effects of (S)-(-)-limonene fumigation followed by ethylene exposure on cumulative flowers/buds abscission in different varieties s of waxflower.

The antagonistic effects of (*S*)-(-)-limonene (1 µM) fumigation, ethylene (10 μLL^{-1}) alone and in combination on cumulative abscission of flowers/buds over a period of four days after treatment in 'WX73', 'WXFU', 'WX17', 'Purple Pride', 'WX56' and 'WX58' waxflowers were evaluated over four days during 2014 and 2015 (Fig. 3 and 4). The flower sprigs of 'WX73' and 'WXFU' fumigated with 1 µM (*S*)-(-)-limonene and followed by exposure to ethylene (10 μLL^{-1}) for 24 h showed significantly reduced cumulative flowers/buds abscission from day one to four (19.3% to 31.1 and 26.8 to 33.8%) in 'WX73' and 'WXFU' respectively in 2014 as compared to those treated with 10 μLL^{-1} ethylene alone (86.2% to 91.3% and 79.1 to 83.9% respectively) (Fig.

Table 1. The percentage of open flowers on stems at the time of harvest 2014-2015.

Year	Genotypes	Flowers open on stems (%)
2014	'WX73'	81.8±11.2
2014	'WXFU'	95.7±3.4
2014	'WX17'	67.7±14.2
2015	'Purple Pride'	62.2±9.9
2015	'WX56'	74.5±13.8
2015	'WX58'	79.4±5.1

± = Standard error of the mean (SE)

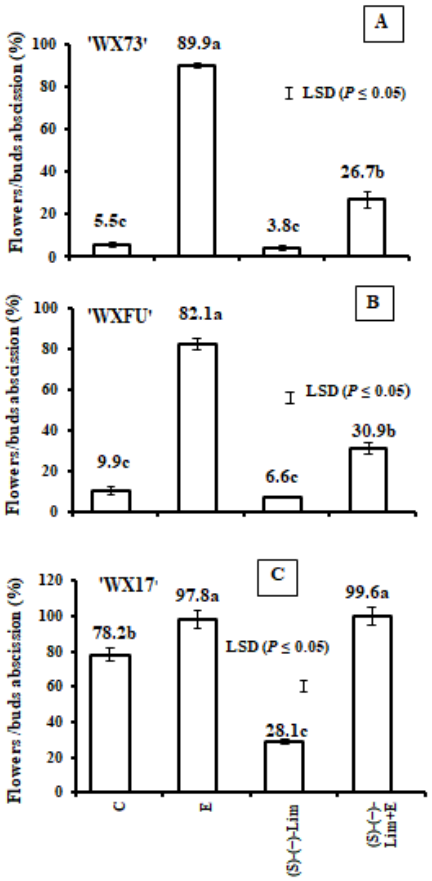

Fig 1. Effects of fumigation of (*S*)-(-)-limonene (1 µM), ethylene (10 µLL^{-1}) alone and (*S*)-(−)-limonene followed by exposure to ethylene (10 µLL^{-1}) on mean flowers/buds abscission in (A) 'WX73', (B) 'WXFU' and (C) 'WX17' waxflower in 2014. Vertical bars represent SE. C = control, E = ethylene alone (10 µLL^{-1}), (*S*)-(-)-Lim = (*S*)-(-)-limonene alone, (*S*)-(−)-Lim + E = (*S*)-(-)-limonene followed by ethylene.

3). Meanwhile, lowest cumulative of flowers/buds abscission was shown on the 'WX73' and 'WXFU' stems treated with the (*S*)-(-)-limonene (1 µM) alone for 18 h (3.1% to 5.1% and 3.4% to 9.6% respectively) as compared to all other treatments and control (Fig. 3). However, cumulative flowers/buds abscission from day one to four in 'WX17' waxflower stems fumigated with (*S*)-(-)-limonene (1 µM) followed by ethylene exposure (10 µLL^{-1}) did not differ significantly from the flower stems treated with ethylene exposure (10 µLL^{-1}) alone (Fig. 3).

The highest cumulative abscission of flowers/buds was observed on 'Purple Pride', 'WX56' and 'WX58' branches when fumigated with the ethylene treatment alone from day one to day four (75.5% to 79.1%, 83.9% to 86.7% and 57.6% to 74.6% respectively) as compared to the sprigs treated with 1 µM (*S*)-(-)-limonene and followed by exposure to ethylene (10 µLL^{-1}) for 24 h (52.7% to 66.3% 'Purple Pride', 11.6% to15.3% 'WX56' and 0.75 to 11.8% 'WX58') in 2015 (Fig. 4). Meanwhile, the (*S*)-(-)-limonene treatment alone showed the lowest cumulative flowers/buds abscission in 'Purple Pride' (0.0% to 1.5%) as compared to the untreated stems (39.8% to 46.5%). Untreated 'WX56' and 'WX58' waxflower stems exhibited the lowest cumulative abscission of flowers/buds as compared to all other treatments (Fig. 4).

Discussion

Postharvest abscission of flowers/buds, petal and leaf senescence in waxflower causes serious economic losses to the Australian waxflower industry (Joyce, 1993; Dinh et al., 2008; Seaton and Poulish, 2010). To avoid the harmful

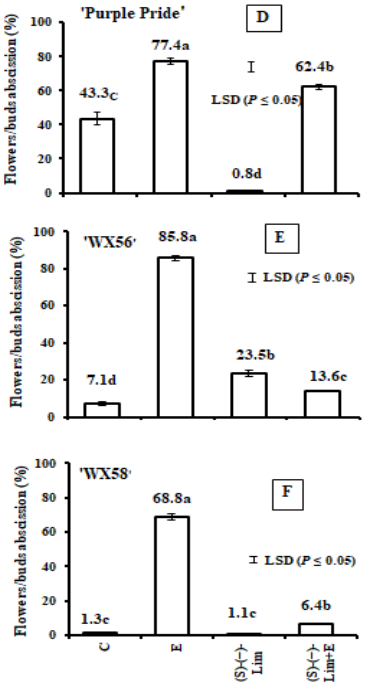

Fig 2. Effects of fumigation of (*S*)-(-)-limonene (1 µM), ethylene (10 µLL^{-1}) alone and (*S*)-(-)-limonene followed by exposure to ethylene (10 µLL^{-1}) on mean flowers/buds abscission in (D)'Purple Pride', (E) 'WX56' and (F)'WX58' waxflower in 2015. Vertical bars represent SE. C = control, E = ethylene alone (10 µLL^{-1}), (*S*)-(-)-Lim = (*S*)-(-)-limonene alone, (*S*)-(-)-Lim + E = (*S*)-(-)-limonene fumigation followed by ethylene.

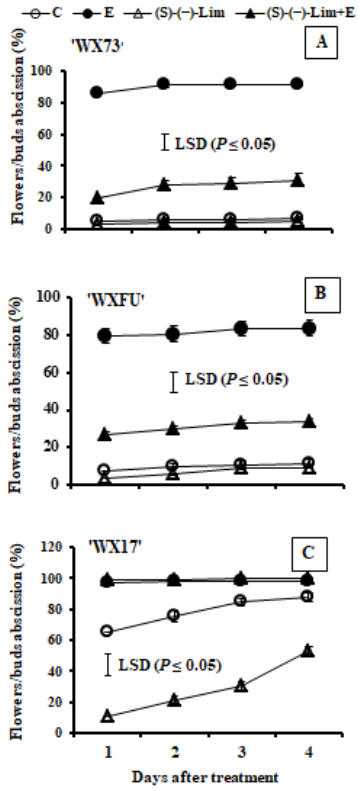

Fig 3. Effects of fumigation of (*S*)-(-)-limonene (1 µM), ethylene (10 µLL^{-1}) alone and (*S*)-(-)-limonene followed by exposure to ethylene (10 µLL^{-1}) on cumulative abscission of flowers/buds over four days after treatment in (A)'WX73', (B)'WXFU' and (C)'WX17' waxflower in 2014. n = three replications (three stems per replication), vertical bars represent SE, C = control, E = ethylene alone (10 µLL^{-1}), (*S*)-(-)-Lim = (*S*)-(-)-limonene alone, (*S*)-(-)-Lim + E = (*S*)-(-)-fumigation followed by ethylene.

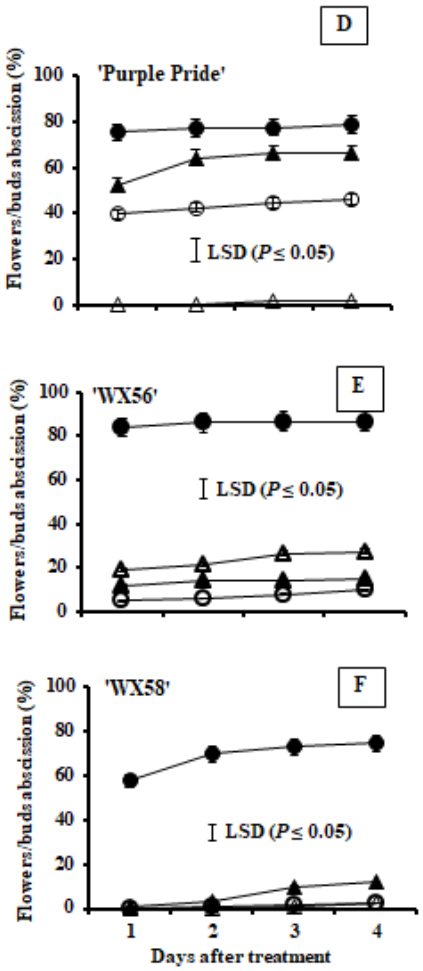

Fig 4. Effects of fumigation of (*S*)-(-)-limonene (1 µM), ethylene (10 µLL^{-1}) alone and (*S*)-(-)-limonene followed by exposure to ethylene (10 µLL^{-1}) on cumulative abscission of flowers/buds over four days after treatment in (D)'Purple Pride', (E) 'WX56' and (F)'WX58' waxflower in 2014. n = three replications (three stems per replication), vertical bars represent SE, C = control, E = ethylene alone (10 µLL^{-1}), (*S*)-(-)-Lim = (*S*)-(-)-limonene alone, (*S*)-(-)-Lim + E = (*S*)-(-)-limonene fumigation followed by ethylene.

effects of exposure to unfavourable conditions that accelerate ethylene biosynthesis or render the flower more sensitive to ethylene may be an effective approach to reduce postharvest losses in horticulture produce including flowers (Kader, 2003; Ebrahimzadeh et al., 2008; Scariot et al., 2014). As a prelude, application of different ethylene antagonists such as STS and 1-MCP are effective in reducing damaging effects of ethylene in horticultural crops but, are coupled with some weaknesses (Blankenship, 2001; Blankenship and Dole, 2003; Kader, 2003; Seaton and Poulish, 2010).

Fumigation of (*S*)-(-)-limonene (1 µM) for 18 h followed by ethylene exposure (10 µLL^{-1}) for 24 h has substantially lowered flowers/buds abscission on 'WX73', 'WXFU', 'Purple Pride', 'WX56' and 'WX58' waxflower during 2014 and 2015 (Fig.1 and Fig. 2) as compared to those treated with ethylene alone. Significant reduction in abscission of flowers/buds was shown in different genotypes of waxflower when stems were treated with (*S*)-(-)-limonene followed by exposure to ethylene. This can probably be ascribed to the inhibitory activity of applied (*S*)-(-)-limonene on ethylene action in waxflower. The experimental data suggest that (*S*)-(-)-limonene treatment seems to be very effective in blocking the ethylene action in waxflower consequently reducing the abscission of flowers/buds. Possibly, (*S*)-(-)-limonene seems to be binding to the ethylene receptor on 'WX73', 'WXFU', 'Purple Pride', 'WX56' and 'WX58' waxflower but, the exact mode of action of (*S*)-(-)-limonene as an ethylene antagonist warrants to be further investigated. Previously, by Gricko et al. (2003) also reported the existence of one double bond as functional groups in the chemical structure one inside the ring and the other outside the ring. Possibly, the interaction of the double bonds in this chemical structure with the ethylene receptor site may make these compounds active in masking ethylene receptor sites in flowers.

Meanwhile, no such reduction in abscission of flowers/buds in 'WX17' in 2014 was noted when the stems were treated with (*S*)-(-)-limonene followed ethylene exposure and with ethylene alone (Fig. 1). This differential response to the application of (*S*)-(-)-limonene followed by ethylene exposure and with ethylene alone may be attributed to the genetic variation among different genotypes of waxflower. Similarly, the sensitivity of waxflower to ethylene exposure appeared to be a genetic trait particularly ascribed to the signal transduction pathways and/or the number as well as the affinity of ethylene receptors in the floral tissues (Tieman and Klee, 1999; Macnish et al., 2004a). (*S*)-(-)-limonene also has potential as a postharvest treatment to prevent flower/buds abscission in waxflower. Variation among waxflowers genotypes may require further

investigation to ensure that it is effective for varieties of waxflower being treated as well as concentration used. Also, the degree of ethylene control needs to be at a sufficient level to be considered as a replacement for other methods such as STS and 1-MCP.

Materials and Methods

Source of chemicals

(*S*)-(-)-limonene was purchased from Sigma-Aldrich, Castle Hill, NSW, Australia. Ethylene gas (98%) was procured from BOC Gases, Australia Ltd., Perth, Australia.

Plant material

The flower stems of *Chamelaucium* varieties such as 'WX73' (*Chamelaucium uncinatum* Schauer. x *Verticordia grandis* Desf.), 'WXFU' (*C. uncinatum* Schauer. x C. sp. Walpole (P.G.Wilson 6318), 'WX17' and 'Purple Pride' (*C. uncinatum* Schauer.), 'WX56' and 'WX58' (*C. uncinatum* Schauer. x C. *megalopetalum* (F. Muell. ex Benth.) were harvested from five-year-old bushes which were grown under irrigation and fertigation (Seaton and Poulish, 2010) at the Department of Primary Industries and Regional Development, (DPIRD), South Perth. Six separate experiments were conducted during the winter-to-spring (June to October) in flowering season 2014 and 2015 to evaluate the effects of (*S*)-(-)-limonene as an ethylene antagonist on fresh cut waxflower stems of 'WX73', 'WXFU' and 'WX17' during 2014 and 'Purple Pride', 'WX56' and 'WX58' in 2015.

Harvesting flowering stems

The flowering stems of six varieties 'WX73', 'WXFU', 'WX17' and 'Purple Pride', 'WX56' and 'WX58' were picked (60 to 70 cm in length) in the early morning and immediately placed in buckets of clean tap water. At the laboratory, stems were completely randomised by spreading out on bench to mix stems and then recut under water to a length of 30cm to avoid air embolism prior to the application of different treatments. Table 1 presents the percentage of flowers open per stem for in six genotypes tested including a standard error of the mean (SE).

Treatments and methods

During the experimental period, in all the treatments the flower sprigs were kept in 250 ml small translucent plastic vases containing distilled water. Flower stems kept in distilled water with no fumigation treatment were assigned as a control. Flower branches were fumigated for 24 h with ethylene (10 μLL^{-1}) alone (*S*)-(-)-limonene (1 µM) alone for 18 h and (*S*)-(-)-limonene (1 µM) for 18 h followed by exposure to ethylene (10 μLL^{-1}) 24 h. The (*S*)-(-)-limonene compound was applied (1µM) with the flower stems on filter papers in petri dishes inside the 60 L plastic drums. All other treatments were applied to flower stems in the plastic drums.

Experiments

Effect of fumigation of (S)-(-)-limonene, ethylene alone and (S)-(-)-limonene treated flowers followed by ethylene exposure on abscission of flowers/buds of 'WX73' 'WXFU', 'WX17', 'Purple Pride', 'WX56' and 'WX58' varieties of waxflowers in 2014 - 2015.

Antagonistic effect of (*S*)-(-)-limonene was tested in three independent experiments using 'WX73', 'WXFU' and 'WX17' in 2014 and three more independent experiments in 2015 on 'Purple Pride', 'WX56' and 'WX58'. After the termination of the 24 h of ethylene treatment the flower bunches were taken out of the vases and softly beaten on the table to collect the abscised flowers/buds. Flowers/buds abscission was recorded daily following the treatments for four consecutive days. Cumulative flowers/buds abscission during the four days was calculated and expressed as a percentage of total abscised and intact flowers/buds.

Experimental design and statistical analysis of data

All the six experiments were laid out by following two-factor factorial completely randomised design including the (treatment and time) with three replications and three stems were treated as an experimental unit. The data were subjected to a two-way analysis of variance (ANOVA) by employing GenStat 16th edition (Lawes Agricultural Trust, Rothamsted Experimental Station, Rothamsted, UK). Treatment means were compared by LSD at ($P < 0.05$) and means (± SE) shown as appropriate. Differences among treatments were compared using Duncan's Multiple Range Test.

Conclusion

A substantial reduction in abscission of flowers/buds in five 'WX73' 'WXFU', 'Purple Pride', 'WX56' and 'WX58'different varieties of six waxflower treated with (*S*)-(-)-limonene prior to the exposure to ethylene suggests that it acts as an ethylene antagonist.

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