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Short Communication

ALKAMIDE-RICH *HELIOPSIS LONGIPES* EXTRACT PROMOTES RESISTANCE TO GREY MOULD DISEASE IN ORNAMENTAL LISIANTHUS

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SUMMARY

Lisianthus is an important flower crop susceptible to *Botrytis cinerea* grey mould. Therefore we studied whether lisianthus plants could be protected with the use of alkamides from the herb *Heliopsis longipes* against artificially inoculated *B. cinerea* during pre- and postharvest. Grey mould symptoms on inoculated leaves of lisianthus were decreased three times by pre-treatment with alkamides, compared to untreated, greenhouse-cultured lisianthus. In postharvest stored shoots no protection was observed. Our results show that the exogenous application of alkamides promoted resistance in lisianthus plants against *B. cinerea* during preharvest but not postharvest storage.

Keywords: *Botrytis cinerea*, *Eustoma grandiflorum*, *Heliopsis longipes*, preharvest, postharvest.

Lisianthus [*Eustoma grandiflorum* (Raf.) Shinners] is an economically important ornamental crop plant (Harbaugh, 2006) from the Gentianaceae family and it is native to grasslands from southern North America to northern South America (Kawabata *et al*., 2012). In 2014, the production of lisianthus in the United States was worth $3.84 million, with California accounting for 90.63% of those sales (USDA, 2015). Given the high value in cut-flower market, major economic losses are often linked to the grey mould disease on lisianthus caused by *Botrytis cinerea* Pers. (Wolcan *et al*., 1996; Vrind, 2005). The symptoms related to grey mould on *E. grandiflorum* comprise crown rot, damping off, stem cankers, and stem, leaf, and flower blight; grey mould can cause postharvest deterioration of flowers by damaging petals (Wegulo and Vilchez, 2007). Several studies have been performed on the lisianthus-grey mould pathosystem. Vrind (2005) found that *E. grandiflorum* was more susceptible to *B. cinerea* infection compared to rose and gerbera plants. Wegulo and Vilchez (2007) used various techniques to evaluate 12 lisianthus cultivars for resistance to grey mould and found that some commercial cultivars are moderately resistant to *B. cinerea* in growth chambers and greenhouse conditions. In another study, Shpialter *et al.* (2009) demonstrated that grey mould development was favoured by air temperatures between 15 and 20°C and 85 to 99% relative humidity (RH); they also observed significantly reduced disease severity at 26°C and 70-85% RH, and when calcium was supplied through fertigation. In addition, plastic mulching with silver/black coextruded polyethylene film also suppressed grey mould considerably in lisianthus soil culture (Shpialter *et al*., 2009).

In greenhouse production, *B. cinerea* is considered one of the most important fungal pathogens negatively affecting lisianthus yields (Vrind, 2005), therefore it is essential to use different strategies for integrated control and to enhance resistance to grey mould disease. Among such alternatives, alkamide-rich root extracts could represent a sustainable approach to crop protection (Molina-Torres *et al*., 2004; López-Bucio *et al*., 2013). Alkamides are metabolites with a broad range of biological activities; Ramírez-Chávez *et al.* (2004) reported increased plant growth and early root development in *Arabidopsis thaliana* when it was treated with affinin, a major alkamide produced by the herb *Heliopsis longipes* S.F. Blake. Furthermore, alkamides application induced changes in expression of genes encoding for jasmonic acid (JA) biosynthesis, nitric oxide (NO) and H_2O_2 accumulation, thus conferring resistance against *B. cinerea* (Méndez-Bravo *et al*., 2011).

To evaluate the resistance in lisianthus against *B. cinerea*, seedlings of lisianthus (*Eustoma grandiflorum* cv. Mariachi Blue) were grown in 1.3 l pots containing peat moss:perlite (70:30 v/v) and were maintained under greenhouse conditions on November 2014 and April 2015 in Saltillo, Coahuila, Mexico. The plants were watered

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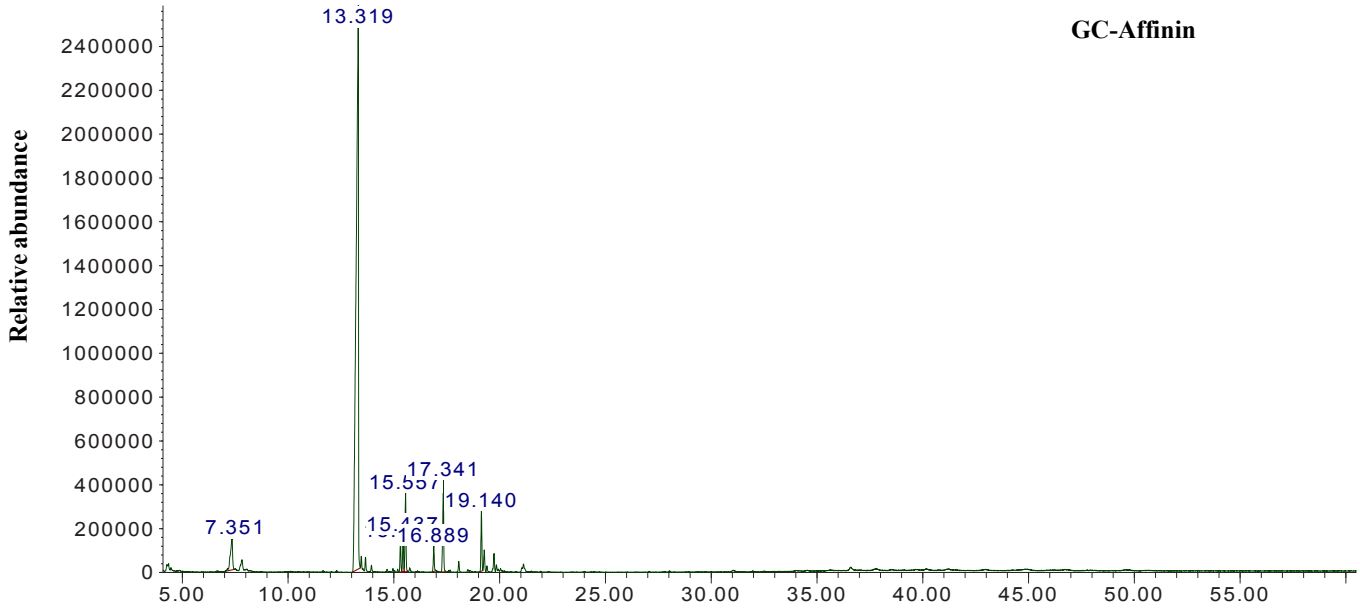

Fig. 1. Chromatogram by GC/MS analysis showing affinin (RT = 13.319 min) as the most abundant alkamide compound in the crude extract from *Heliopsis longipes* roots.

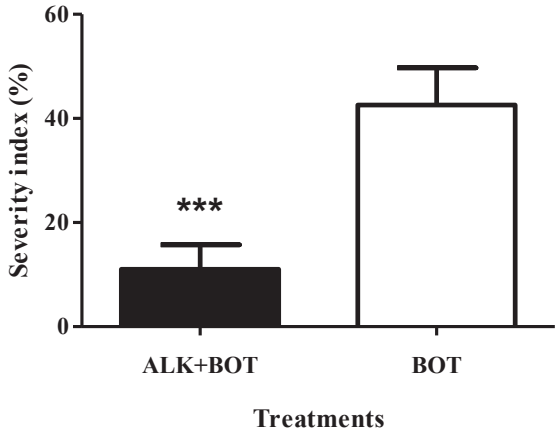

Fig. 2. Disease severity of *Botrytis cinerea* Pers. in lisianthus plants treated (ALK+BOT) or not (BOT) with an alkamide-rich extract 24 h prior to inoculation. Severity index is expressed by leaf area percentage showing symptoms. Significant difference between treatments by *t*-test analysis ($P = 0.0004$).

every third day, and fertilized once a week with 500 ml of a 2 g/l 20-10-20 N-P-K water-soluble fertilizer (Peat-Lite-Special; Peters Professional; Scotts-Sierra Horticultural Products Co, Marysville, OH); young plants approximately 15 cm tall were employed for all resistance assays. The alkamide-rich extract was obtained from *H. longipes* as described by Ramírez-Chávez *et al.* (2004); briefly, the roots (1 kg) were ground and extracted in 10 l of ethanol at room temperature for 1 week, later the alcoholic extract was evaporated to dryness, dissolved in hexane and fractionated in a 50-cm open glass column packed with silica gel mesh 200 (J.T. Baker, Paris, KY); 50 ml from the fraction eluted with hexane:ethyl acetate (70:30) was collected, evaporated and dissolved in 1 ml of ethanol (HPLC degree) and analysed by gas chromatography coupled to a mass selective detector (GC/MS) (model 5890, Hewlett-Packard, Palo Alto, CA) (Fig. 1). The affinin was quantified from fractions eluted according to Molina-Torres *et al.* (1996) and purified by column chromatography and three times by thin-layer chromatography (TLC) to obtain one main peak on GC/MS (data not shown). The affinin extract was separated by TLC until reached 100% of purity and finally, the calibration curve of affinin was performed according to Ramírez-Chávez *et al.* (2004). The commercial standard of pure affinin was not available. One hundred and fifty microliters of the root concentrated extract (1000 ml in total, 78 g of affinin/l) was delivered by dripping on the stem base of lisianthus before pathogen inoculation. To evaluate alkamide-rich extract effects, one leaf from the third knot and one from the fourth knot counting from the base of lisianthus plants were transversally cut (0.5 cm long cuttings) with a new sterile scalpel blade, and then inoculated with 10 µl conidial suspension (1×10^8 spores/ml) obtained from *B. cinerea* 5-day-old PDA cultures (10 cm Petri plates incubated at 25°C in darkness). Ten days following inoculation, the plants were evaluated for grey mould disease, on a 0 to 100% scale according to the percentage of leaf area showing blight, leaf necrosis or reproductive mycelia (Fig. 2). The experiments were performed by randomized block design with three treatments (control, *B. cinerea* and alkamides-*B. cinerea*) and the data were evaluated statistically by t-test analysis (GraphPad Prism version 5 for Windows, GraphPad Software, La Jolla, California USA). Since Ramírez-Chávez *et al.* (2004) reported that alkamides promoted arabidopsis growth, an additional experiment was performed to assess the possibility that treatment by alkamide-rich extracts

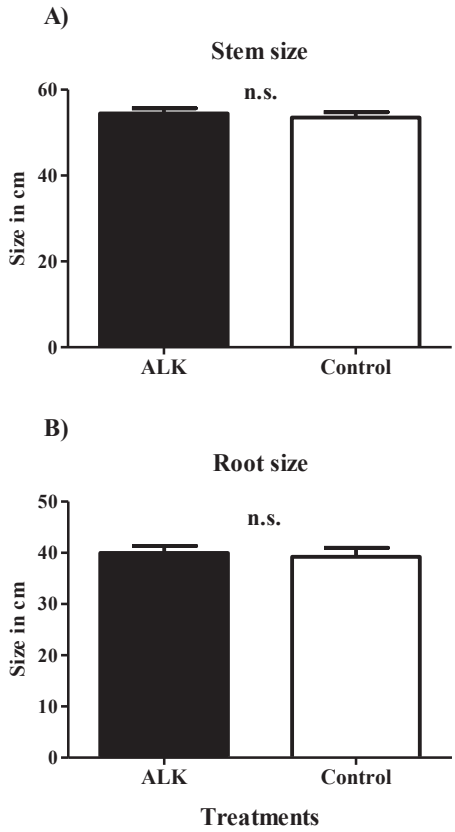

Fig. 3. Growth promotion assay in lisianthus plants performed in greenhouse conditions with an alkamide-rich extract (ALK) or without (Control) treatment. Stems (A) and roots size (B) were evaluated two weeks after alkamides treatment; no significant differences were detected by *t*-test analysis (n.s.).

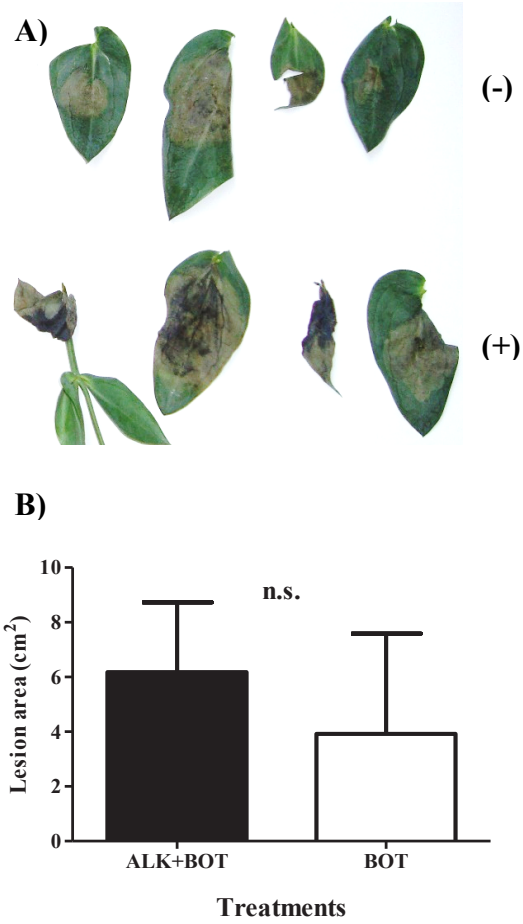

Fig. 4. Postharvest test on cut flowering shoots of lisianthus. (A) Grey mould symptoms in plants with an alkamide-rich extract (+) or without (–) treatment. (B) Lesion area on leaves by *B. cinerea* with alkamides treatment (ALK+BOT) and not treated (BOT), twenty-one days after postharvest inoculations and storing at 7 ± 1°C. No significant differences were detected by *t*-test analysis (n.s.).

might influence vegetative growth of lisianthus plants; however, no growth promotion was observed (Fig. 3).

For postharvest testing, the plants were grown for 100 days after transplanting; alkamides were applied to the stem base once, 90 days after transplanting, during anthesis but prior to blooming. Ten days later, flowering shoots with one to three open flowers were cut 10 to 15 cm above soil level. Then the stems were placed in 20 l PVC containers with five liters of distilled water, and stored in a cold chamber (7 ± 1°C; 80% RH). One day after the beginning of cold storage, two leaves from each stem, one fully expanded close to the base, and one not fully expanded close to the apex, were injured in the same way as in the preharvest assay and inoculated as described above. Twenty-one days following inoculation, the plants were evaluated for grey mould disease as indicated before (Fig. 4).

Our results are similar to those reported by Méndez-Bravo *et al.* (2011) in which alkamide treatment induced resistance in *A. thaliana* against *B. cinerea*, resistance that is mediated by JA-dependent responses, among others. In spite of the positive impact of the alkamide-rich extract on *E. grandiflorum* resistance to grey mould disease during preharvest, the postharvest tests showed that the alkamide-rich extract did not protect cut flowering shoots and the leaves presented abundant typical symptoms of grey mould. This could be explained by the fact that mature plants cannot activate the defence responses in the same way as young plants do (Dinh *et al.*, 2007). Furthermore, *B. cinerea* activity has been observed at temperatures as low as 0°C, as Droby and Lichter (2004) reported. In conclusion, our results suggest that the *H. longipes* alkamides application could represent an environmentally friendly alternative to control lisianthus fungal diseases, but treatment timing with regard to the physiological stage should be carefully studied.

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