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BRIEF COMMUNICATION

Volatile monoterpenes from *Prinsepia utilis* L. leaves inhibit stomatal opening in *Vicia faba* L.

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Abstract

Volatile essential oils from *Prinsepia utilis* L. have been shown to inhibit stomatal opening in *Vicia faba* L. epidermal peels as well as whole leaves as shown by measurement of pore width and stomatal conductance (g_s). The stomatal closure was associated with inhibition of K^+ influx in the guard cells. Gas chromatography/mass spectroscopy analysis of the steam distillate from *P. utilis* L. leaves showed the presence of over twenty compounds; of which thirteen compounds have been identified.

Additional key words: allelopathy, stomatal conductance, volatile essential oils.

Chemical interaction between plants has been studied in some detail and termed as allelopathy. In such studies mostly the effects of chemicals exuded/leached from one plant, have been studied on seed germination (*e.g.* Rice 1984, Narwal 1994). In most cases the compounds leached or exuded are either phenolics or alkaloids (Einhellig 1999). Functions of aromatic compounds emanating from plants has mostly been studied with reference to their action as insect attractants or repellants (Pare and Tumlinson 1999). It is possible that chemicals released in vapour form could also affect other plants. Only few reports show inhibition of seed germination and inhibition of root growth (Muller and Muller 1964, Asplund 1968, Del Moral and Muller 1970). To the best of our knowledge the effect of volatiles released from one plant species on the aerial parts of other plants has never been studied. Stomata constitute the interface between plant interiors and the surrounding atmosphere. It is conceivable that stomata will respond to the released volatile compounds by neighboring plants and that the consequences of altered stomatal movements could be considerable for plant growth. *Prinsepia* was chosen since it shows wide allelopathic potential in seed germination studies and *Vicia* stomata is a well established and characterized material for stomatal studies.

Sterilized seeds of *Vicia faba* L. were grown in mixed garden soil. Fully expanded 3rd or 4th leaf from the top from 4 to 5-week-old plants was used. Abaxial epidermal strips were prepared and floated in cavity blocks containing 10 mM PIPES buffer, pH 6.8 (Rai and Sharma 1991). The cavity blocks were enclosed in a transparent Zip-loc bag (28 × 23 cm) containing air-dried *Prinsepia utilis* L. leaves collected from local wild populations (5 or 10 g) separately or 1.0 - 1.5 cm^3 of steam distillate from *Prinsepia* leaf powder on *Whatman no. 1* filter paper, methanol was allowed to evaporate. Such inclusions in Zip-loc bags helped build-up of volatile components within the bag. A bag without leaf constituted the control. The Zip-loc bags were incubated under white fluorescent light (11 W cm^{-2}) at 25 ± 2 °C, for 1 h equilibration and then leaf powder or distillate on filter paper was included in the bag for 24 h. Finally, epidermal peels were fixed in EtOH and mounted in Heath's reagent. Stomatal pore width was measured using a calibrated eye piece graticule. From nine strips each, 15 random measurements were made. Experiments were repeated three times. K^+ in the guard cells was localized histochemically using cobalt nitrite (Laloraya *et al.* 1986). For

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stomatal conductance (g_s) measurements, *V. faba* leaves with petiole dipping in water were included in the bag and after 24 h treatment g_s was measured with a *Licor 1600* (Lincoln, USA) steady state porometer.

Steam distillate was prepared from 40 g of dry leaf powder according to Waller and Feng (1996) and finally dissolved in MetOH (7 cm^3). Gas chromatography/mass spectroscopy (GC/MS) analysis of the steam distillate was done on *Perkin-Elmer* (Norwalk, USA) model *Q-mass 910* equipped with *Supelcowax-10* capillary column. Helium was used as carrier gas (6.4 cm^3 min^{-1}). Identification of compounds was done using computer library search (Stein 1990) and visual interpretation of mass spectra (Jennings and Shibamoto 1988, Adams 1989, McLafferty 1989).

Inclusion of 5 g dry leaf powder in the treatment bag inhibited stomatal opening by *ca.* 30 % which increased to 95 % with 10 g of leaf powder. Experiments were reproducible with inter experimental variation with in 10 % range (Table 1).

Table 1. Effect of dry leaf powder or volatile from the dry leaf distillates of *Prinsepia utilis* L. on stomatal aperture in *Vicia faba* L. epidermal peels and stomatal conductance of *Vicia* leaves (for details see text). Means ± SD of nine replicates of 15 measurements each.

		Aperture [μm] exp. I	exp. II	exp. III	g_s [cm s^{-1}]
Dry leaves	control	10.33 ± 1.70	8.74 ± 1.79	12.09 ± 1.93	-
	5 g	7.28 ± 2.13	5.27 ± 1.56	7.17 ± 2.06	-
	10 g	0.64 ± 1.23	2.08 ± 1.56	2.67 ± 1.92	-
Leaf distillate	control	9.83 ± 1.52	11.27 ± 1.60	10.76 ± 1.94	0.730 ± 0.020
	1.0 cm^3	5.67 ± 1.71	4.72 ± 1.54	5.30 ± 1.45	0.047 ± 0.005
	1.5 cm^3	2.71 ± 2.07	3.29 ± 1.40	2.81 ± 1.55	0.036 ± 0.007

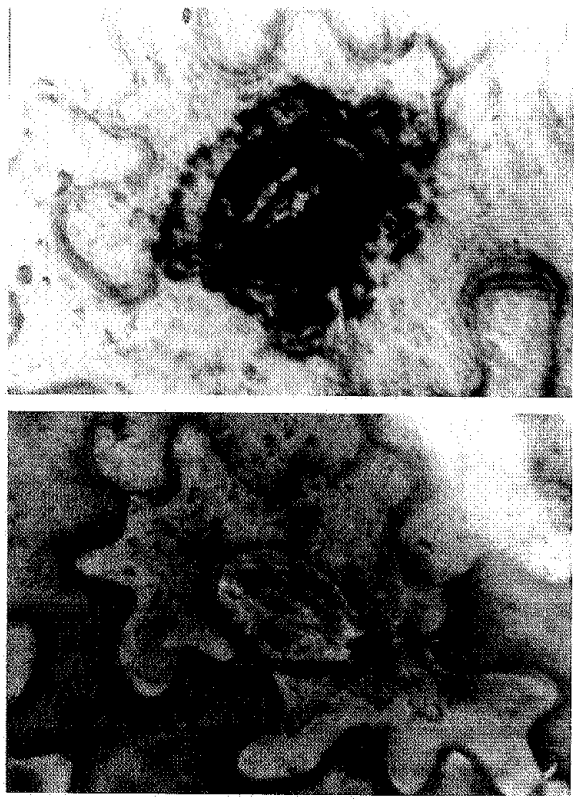

Fig. 1. K^+ staining in guard cells of *V. faba* L. leaf epidermal peels. Upper - control, lower - exposed to volatile from *P. utilis* leaves (90 % stomata showed similar differences).

Since most of the components of volatile nature in plants are essential oils, they were steam distilled and distillate was taken in methanol. The exposure of epidermal peels to varying amounts of distillate led to the inhibition of stomatal opening (Table 1). One and 1.5 cm^3 of distillate caused inhibition of *ca.* 50 and 70 %, respectively. To check if inhibition will also be valid for whole leaves, the leaf g_s measurements were also made (Table 1) and increasing concentrations of volatiles caused a decrease in g_s. Since stomatal opening has been correlated to K^+ fluxes in guard cells, K^+ ions in the guard cells have been observed in control and volatile treated epidermal peels (Fig. 1). The guard cells in controls exhibited an abundance of K^+ (black precipitate) that was drastically reduced due to treatment with volatiles. Thus, stomatal closure by volatiles appears to be directly related to their ability to inhibit K^+ influx into the guard cells.

GC/MS studies showed presence of at least 22 compounds in the steam distillates, out of these 13 could be identified: limonene (0.31 %), 1-8-cineole (2.84 %), *o*-cymene (0.26 %), bergamal (0.64 %), cis-linalooloxide (0.39 %), cis-sabinene hydrate (0.75 %), linalool (50.42 %), trans-terpineol (1.61 %), 2-undecanone (36.77 %), isomenthol (0.88 %), α-terpineol (0.45 %), 2-dodecanol (0.36 %) and tridecanone (1.94 %). The major constituents were linalool and 2-undecanone, others were found in traces only. However, it cannot be concluded as to which one(s) are the active compounds. This needs to be ascertained through studies with pure compounds.

Allelochemical effects on seed germination and seedling growth have been studied in many plants but effects on stomatal opening have been studied rarely. Vikherkova (1970) observed inhibition of stomatal opening and transpiration in *Linum usitatissimum* grown in soil treated with rhizome extracts from *Agropyron repens*. This effect was mediated *via* water-soluble compounds absorbed by *Linum* roots and transported to leaf through transpiration stream. Such aqueous extracts are often rich in phenolic compounds, which could potentially influence the stomatal movements. For example, Zelitch (1967) showed a 50 % inhibition of stomatal opening in tobacco leaf discs floated on chlorogenic acid. Similar inhibition of stomatal opening by phenolics has been confirmed (Einhellig *et al.* 1970, Einhellig and Kuan 1971). However, Rai *et al.* (1986) showed that ten tested phenolics reverted the ABA induced stomatal closure in *Commelina communis*. This was confirmed by Laloraya *et al.* (1986) in maize, bean, onion and *Vicia*. Therefore, if donor plant phenolics reach the leaves of recipient plant, they could show variable levels of interaction. However, that volatiles from donor plants can affect the stomatal functioning of recipient plant is shown here for the first time. Earlier observations of Waller and Feng (1996) did give some indication that such volatile allelochemical interaction could be significant in nature. They observed that if leaves of cotton touched or even came in close proximity (< 6 cm) of devil's claw plant then cotton leaves senesced and eventually died. Thus an aerial interaction was apparent but its mechanism could not be ascertained. In this reference, Sharma *et al.* (1995), have shown that in *Tropaeolum majus* leaves senescence was induced by stomatal closure. ABA, which closed stomata, induced senescence while *t*-cinnamic acid, which partially opened stomata, delayed senescence. Thus it seems that senescence in cotton leaves in Waller and Feng's experiments could be induced by stomatal closure in cotton leaves. Such an aerial chemical interaction in field condition is possible if volatile levels could be raised by close proximity as in the above case or by bushy habit *etc.* To the best of our knowledge, this is the first report of effects of mono-terpenes on stomatal opening. However, effects of terpenes on seed germination and seedling growth have been reported. Muller and Muller (1964) showed that volatile inhibitors from *Salvia leucophylla*, *S. apiana* and *S. mellifera* inhibited root growth of test seedlings and active compounds identified as camphor and cineole in the air around *Salvia* plants. Asplund (1968) studied relationship between structures of ten tested mono-terpenes on radish seed germination and observed that compounds with functional ketonic group camphor and pulegone were more inhibitory than others. α-pinene and cineole have also been recognized as most potent inhibitors in *Eucalyptus camaldulensis* showing allelopathic activity (del Moral and Muller 1970). In the present study, the most active compound remains to be identified. The possibility of linalool, being the dominant one, remains high. The other dominant compound 2-undecanone being ketonic will not be volatile at room temperature. In preliminary experiments with *Eucalyptus* leaves, volatiles emanated did not show stomatal closure in *V. faba* (data not presented). Thus probability of α-pinene and cineole, present in *Eucalyptus*, affecting stomatal closure is very low.

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