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THE ISOPENTENYL ISOFLAVONE LUTEONE AS A PRE-INFECTIONAL ANTIFUNGAL AGENT IN THE GENUS *LUPINUS*

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Key Word Index—Leguminosae; Lotoideae; *Lupinus*; isopentenyl isoflavones; luteone; pre-infectional phenols; antifungal activity; absence of phytoalexins.

Abstract—The prenylated isoflavone luteone has been isolated from healthy leaves of *Lupinus albus* and 11 other lupin species. Evidence is presented that this compound occurs as a leaf surface constituent. *In vitro* tests indicate that luteone and a second unidentified isoflavone from *L. albus* possess antifungal activity sufficient to support their proposed role as pre-infectional resistance factors. No evidence was obtained to suggest that phytoalexins were produced by the fungus-infected leaves of *L. albus*.

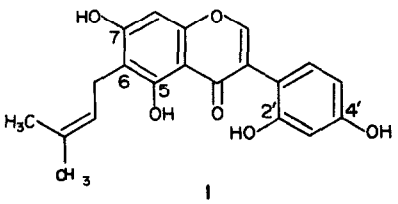
INTRODUCTION

Many higher plants produce *de novo* antifungal compounds or phytoalexins [1] following infection of their leaves and other tissues by non-pathogenic fungi. Phytoalexins associated with the Leguminosae (subfamily Lotoideae) are highly fungitoxic and in affected tissues accumulate to levels which generally exceed those necessary to inhibit fungal growth. There is abundant evidence to suggest that phytoalexins have a primary role in disease resistance although their precise mode of action is still under investigation. However, whilst phytoalexin formation is a widespread phenomenon other chemically-based resistance mechanisms have also been described [2,3].

Of the numerous plant families investigated for phytoalexin biosynthesis [1], the Leguminosae is pre-eminent in its production of reduced isoflavonoids based on the pterocarpan, isoflavan or isoflavanone skeleton. A recent survey of 14 leguminous genera [4] has revealed many isoflavonoid phytoalexins including several hitherto undescribed compounds. In depth studies of several genera showed uniformly positive results; for example, 35 *Trigonella* species produced *de novo* one or other of five isoflavonoid compounds [5]. In contrast, phytoalexins were apparently not produced by the fungus-infected leaves of *Lupinus albus* L. (white lupin); the leaves of this and other *Lupinus* species were characterised by the presence of constitutive antifungal isoflavones. This paper describes the isolation, identification and effect on fungal growth of one of these compounds and presents evidence to suggest that, in the genus *Lupinus*, the occurrence of pre-infectional agents may replace phytoalexin production as a disease resistance mechanism.

RESULTS AND DISCUSSION

No evidence for phytoalexin accumulation was obtained 48 hr after detached leaflets of *L. albus* cv. 'Pflugs Ultra' were inoculated with conidial suspensions (*ca* 5×10^4 spores/ml) of the non-pathogenic fungus, *Helminthosporium carbonum* Ullstrup. Instead, TLC ($CHCl_3$–MeOH, 100:2) bioassays using *Cladosporium herbarum* Fr. [6] indicated that diffusates (see Experimental) from fungus-infected and water-inoculated leaves contained substantial (and approximately equivalent) amounts of two antifungal compounds (LA-1, R_f 0.24; LA-2, R_f 0.12) both of which gave a bright orange colour with diazotised *p*-nitroaniline. Compound LA-2 was subsequently identified as luteone **1** (5,7,2′,4′-tetrahydroxy-6-(3,3-dimethylallyl)-isoflavone) by direct comparison (UV, TLC) with an authentic specimen. Although luteone was first extracted from immature seeds of the yellow lupin (*L. luteus* L.) [7] it has not previously been isolated from *L. albus* or from the leaf tissues of any other *Lupinus* species. The fungitoxicity of luteone has been reported by Fukui *et al.* [7]. Compound LA-1 was not fully identified but from its spectral behaviour was considered to be an isoflavone structurally related to luteone. Although traces of several other isoflavone-like phenolic compounds (diazotised *p*-nitroaniline, orange) were also present in leaf diffusates, they were not antifungal and hence were not further examined. There was no indication that diffusates contained reduced isoflavonoids representative of the pterocarpan, isoflavan or isoflavanone groups.



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Luteone and compound LA-1 were isolated from control and fungus-induced diffusates in approximately similar quantities. Values for luteone (based on log $\epsilon = 4.45$

at 266 nm [7]) in samples from leaves inoculated with water, aqueous 0.05% Tween-20 or spore suspensions of *H. carbonum* were 12, 13 and 13.6 μg/ml respectively. Infection by *H. carbonum* would thus appear to have little or no effect on the general pattern of isoflavone metabolism in *L. albus*. Healthy leaves contained luteone at a concentration of *ca* 30 μg/g. Phytoalexins were not produced when leaves of different ages were inoculated with *H. carbonum*. Furthermore, extracts of infected leaves failed to reveal novel phenolic compounds other than those associated with healthy plants. Leaf washings (obtained by dipping undamaged leaflets in MeOH for 30 sec) also contained luteone and LA-1 thus suggesting that quantities of both compounds were present on the leaf surface.

When incorporated into agar and tested against the mycelial growth of *H. carbonum*, luteone exhibited an ED_{50} value of between 35 and 40 μg/ml. It is thus less active than many pterocarpans and isoflavans [8] but at the same time is considerably more inhibitory to *H. carbonum* than isoflavones (e.g. biochanin A and formononetin-ED_{50} 250–350 μg/ml [4]) which lack an isopentenyl substituent. This attachment may enhance the lipid solubility (and hence the fungitoxicity) of **1** or, alternatively, may confer an essential degree of non-planarity to the luteone molecule [9]. Chromatogram bioassays indicated that luteone (5 μg) was highly inhibitory to *C. herbarum* [6]. In contrast, no antifungal activity was associated with much higher levels (20 μg) of the isoflavones, biochanin A and afrormosin. Fukui *et al.* [7] reported that conidial germination of *Helminthosporium oryzae* van Breda was reduced by 50% at a luteone concentration of *ca* 8 μg/ml.

Histological studies undertaken 48 hr after fungal inoculation revealed that on leaves of barley (*Hordeum* sp.) and maize (*Zea mays* L. cv. 'Seneca Chief') conidia of *H. carbonum* germinated rapidly and produced straight, rarely-branching germ tubes. On leaves of *L. albus* spore germination was apparently not prevented but the resulting germ tubes were curled, distorted and highly branched. A similar antifungal effect has been noted [10] for the sclareol/13-epi-sclareol mixture present on the leaf surface of *Nicotiana glutinosa* L. The occurrence of luteone and LA-1 as leaf surface constituents of *L. albus* suggests that these compounds may primarily influence fungal spore germination and/or germ tube development rather than mycelial proliferation. Under natural conditions the isoflavone concentration in the leaf-surface moisture film might present an *in vivo* environment highly unfavourable for fungal development. This possibility has also been considered by Topps and Wain [11].

Luteone was also isolated from the healthy, undamaged leaves of 11 other lupin species (see *Experimental*) although it was not obtained from *L. densiflorus*. In several instances (e.g. *L. polyphyllus*) luteone was accompanied by LA-1 and other presumably related isoflavones. Although the 7-glucoside of genistein has been identified in flowers of *L. polyphyllus* [12] there was no evidence to suggest its presence in leaf diffusates from this or any other *Lupinus* species. Several compounds with genistein-like UV spectra have been isolated from the roots of *L. luteus* [13]. The unidentified lupin isoflavones isolated during the present study differed in R_f and UV spectra from the common hydroxyisoflavones and appear likely to be structurally similar to luteone.

The results presented above suggest that *Lupinus* differs from most other legume genera in lacking the ability to synthesise phytoalexins following fungal inoculation. Instead, several species from this agriculturally important genus produce luteone, a constitutive isoflavone which appears sufficiently fungitoxic to significantly influence disease resistance. On this basis, luteone (and the second antifungal isoflavone from *L. albus*) could be regarded as a prohibitin [3] together with certain other secondary metabolites previously implicated as pre-infectional disease resistance factors. These include the triterpene avenacin of oats, the guanidino derivatives hordatine A and B of barley, the alkaloid berberine of *Mahonia* sp. and the low-molecular weight phenols, catechol and protocatechuic acid of coloured onions [3]. Although lupin tissues often contain various alkaloids, the possible involvement, in disease resistance, of these compounds was largely excluded by the use of low-alkaloid cultivars of both *L. albus* and *L. luteus*. From the above data, a more extensive investigation of resistance mechanisms in genera related to *Lupinus* (e.g. *Laburnum a* and *Genista*) would appear to be worthwhile.

EXPERIMENTAL

Plant material. Lupin seeds (obtained from various European institutes) were grown (20°, 6500 lx) in a light potting compost. Flower buds were removed regularly to encourage branching and leaf production. In general, experiments involved leaflets removed from plants 3–4 months old. The following species were investigated: *L. albus* cv. 'Pflugs Ultra' and 'Sweet White' *L. arboreus* Sims, *L. cruckshankii* Hook., *L. densiflorus* Benth., *L. hartwegii* Lindl., *L. luteus* L. cv 'Sweet Yellow', *L. micranthus* Guss., *L. mutabilis* Sweet, *L. nanus* Dougl., *L. polyphyllus* Lindl., *L. pubescens* Benth., *L. rivularis* Dougl. and *L. subcarnosus* Hook.

Isolation of isoflavones. Detached leaflets were inoculated with droplets of water, 0.05% aqueous Tween-20 or spore suspensions of *H. carbonum* as previously described [5,14]. TLC (Si gel, $CHCl_3$–MeOH, 100:2) of diffusate [14] extracts (EtOAc) gave luteone (R_f 0.15) and compound LA-1 (R_f 0.32). Elution and subsequent TLC purification (*n*-pentane–Et_2O–HOAc, 75:25:4) afforded **1** (R_f 0.14) and LA-1 (R_f 0.28) as chromatographically homogeneous bands.

Isoflavone identification. Luteone (**1**) was identified by UV and TLC comparison with a sample kindly provided by K. Koshimizu. The spectral properties were in close agreement with the lit [7] values. TLC comparisons (Si gel, 20°, saturated atmos) gave the following R_f values:- Et_2O–*n*-hexane, 3:1, 0.26; $CHCl_3$–MeOH, 100:2, 0.16; $CHCl_3$–HOAc, 5:2, 0.66; *n*-pentane–Et_2O–HOAc, 75:25:1, 0.04; *n*-pentane–Et_2O–HOAc, 75:25:4, 0.11; and C_6H_6–MeOH, 9:1, 0.25. Compound LA-1 gave λ_{max}, MeOH 268 nm; NaOH 281, 340 nm; NaOAc 270, 340 nm; NaOAc + Borate 268 nm; $AlCl_3$ 268, 360 nm; AlC_3 + HCl 269, 360 nm. A third isoflavone (R_f 0.05, $CHCl_3$–MeOH, 100:2) from *L. albus* 'Pflugs Ultra' had λ_{max}, MeOH 262, 290 (*sh*), 330 nm; NaOH 272, 330 nm; NaOAc 272, 338 (*sh*) nm; NaOAc + Borate 262, 290 (*sh*) nm; $AlCl_3$ 270, 310 (*sh*), 365 nm; $AlCl_3$ + HCl 270, 310 (*sh*), 365 nm.

REFERENCES

1. Ingham, J. L. (1972) *Botan. Rev.* **38**, 343.
2. Wood, R. K. S. (1967) *Physiological Plant Pathology* Blackwells, Oxford.
3. Ingham, J. L. (1973) *Phytopath. Z.* 78, 314.
4. Ingham, J. L. (1976) Ph.D. thesis, University of Reading.
5. Ingham, J. L. and Harborne, J. B. (1976) *Nature, Lond.* **260**, 241.
6. Homans, A. L. and Fuchs, A. (1970) *J. Chromatog.* **51**, 327.

7. Fukui, H., Egawa, H., Koshimizu, K. and Mitsui, T. (1973) *Agr. Biol. Chem.* **37,** 417.
8. Ingham, J. L. and Millar, R. L. (1973) *Nature, Lond.* **242,** 125.
9. Perrin, D. R. and Cruickshank, I. A. M. (1969) *Phytochemistry* **8,** 971.
10. Bailey, J. A., Vincent, G. G. and Burden, R. S. (1974) *J. Gen. Microbiol.* **85,** 57.
11. Topps, J. H. and Wain, R. L. (1957) *Nature, Lond.* **179,** 652.
12. Hörhammer, L. and Wagner, H. (1962) *Arzneimittel-Forsch.* **12,** 1002.
13. Volynets, A. P., Mashtakov, S. M. and Laman, N. A. (1969) *Soviet Plant Physiol.* **16,** 779.
14. Higgins, V. J. and Millar, R. L. (1968) *Phytopathology* **58,** 1377.