Proceedings
The 4th International
Carnivorous Plant Conference
Tokyo, Japan 2002 ©

G-1

Secretion of digestive enzymes in *Plumbago*

A. Stoltzfus, J. Suda, R. Kettering, A. Wolfe and S. Williams

Biology Department, Lebanon Valley College, Annville, PA 17003-0501, U.S.A.

Abstract. Phylogenetic studies, using molecular characters, indicate that *Plumbago* (Plumbaginaceae) is related to *Drosera* (Droseraceae) and Drosophyllum (Droserophyllaceae). Sepals of *Plumbago auriculata* Lam. and *Plumbago indica* L. bear large mucilage secreting trichomes, which resemble those of *Drosophyllum*. These trichomes are capable of capturing insects. We used the substrate film technique to test whether *Plumbago* trichomes can be stimulated to secrete proteolytic enzymes by the same stimuli that trigger secretion in *Drosera*. Several different stimuli were tested. There was strong secretion of proteases in response to 5 mM NaCl and NH_4Cl and a weaker response to 5 mM KCl. Yeast extract, which is highly stimulatory of protease secretion in *Drosera* fails to stimulate secretion in Plumbago. *Plumbago auriculata* and *Plumbago indica* are capable of secreting proteases in response to chemical stimulation but do not respond identically to all chemical stimuli effective with *Drosera*.

Introduction

Phylogenetic studies using molecular characters indicate that *Plumbago* (Plumbaginaceae) is related to *Drosera* (Droseraceae), *Drosophyllum*, *Triphyophyllum*, *Nepenthes* and a number of related noncarnivorous genera (Fig. 1; Albert *et al.*, 1992; Williams *et al.*, 1994; Lledo *et al.*, 1998; Meimberg *et al.*, 1999). Sepals of *Plumbago auriculata* Lam. (=*P. capensis* Thunb.) and *Plumbago indica* L. bear large mucilage secreting trichomes, which resemble those of *Drosophyllum* (Rashmilevitz and Joel, 1976). If *Plumbago* is carnivorous it would extend the carnivorous syndrome to groups basal to the clade of caryophyllacious carnivorous plants that has been revealed by cladistic analysis of molecular characters (Fig. 1). The objective of this study is to observe if *Plumbago* trichomes are capable of capturing insects and if they are capable of being stimulated to produce digestive enzymes.

Materials and Methods

Plant materials consisted of *Plumbago auriculata* (=*capensis*) and *Plumbago indica* were from Logee's Greenhouses, Danielson, CT, U.S.A. grown in a greenhouse in a 14 hr daylength.

The substrate film method (Hartmeyer, 1997) as modified by Carroll and Darnowski (2001) at Washington College for use on trigger plants was adapted for use on *Plumbago* flowers. On the first day of the three-day procedure the sepal was stimulated by brushing on the solution that was being tested. Each side of the calex was stroked twice with a freshly dipped brush. After stimulation with NaCl, NH_4Cl, and KCl and yeast extract solutions, the plants were left in the greenhouse for 24 hr. On the second day the flowers were clipped at the base of the pedicle and laid on a piece of Kodak 400 film which had previously been exposed to fluorescent light for a brief time then professionally developed. Just previous to the experiment the segments of film were coated with a solution of KH_2PO_4 buffer to prevent acid hydrolysis of the gelatin emulation. The flower and sepal were applied to the film in a Petri dish, containing a moist paper towel and the Petri dish was sealed with Parafilm and placed under fluorescent lights for 24 hr. The third day the film was removed form the dish

and dipped in tap water to remove any digested gelatin after which the film was allowed to dry. Negative controls involved placing drops of KCl, NaCl, NH_4Cl solutions and double distilled water on the gelatin emulsion film. Positive controls used with *Drosera capensis*, known to secrete proteolytic enzymes. The quantity of gelatin digested from the film surface indicated the quantity of proteolytic enzymes secreted.

Paraffin and plastic embedded sections of *Plumbago auriculata* were produced by cutting the sepals in half longitudinally with a razor blade and fixing them. Paraffin sections were made using Forssmann's procedure (Forssmann *et al.*, 1977). Plastic embedded sections were made by fixing using Kelley's OTO procedure (Kelley *et al.*, 1973), embedding in JB4 Historesin blocks and sectioning with an LKB microtome. The sections were heat fixed then stained. Fresh tissue was frozen and sectioned in a cryostat. Sections were stained at two times that recommended for animal tissue with PAS-Shift's reagent, alcian blue, safranin, crystal violet, toluadine blue, Delafield's hematoxylin and orange G.

Results

Capture of insects occurs on the trichomes of both *Plumbago auriculata* and *Plumbago indica*. We have observed and photographed captured insects both in the field in Florida and in the greenhouse (Fig 2). Observations are limited and it is not certain how many insects are captured but it is clear that insects are captured by the trichomes on *Plumbago* sepals. Insects observed on the trichomes were: ants, spiders, gnats, and flies.

Protease secretion from trichomes on sepals of *Plumbago auriculata* and *Plumbago indica* occurs in response to stimulation by several salts. However, yeast extract, which is capable of stimulating secretion of proteases in both *Drosera* and *Stylidium* trichomes (Carroll and Darnowski, 2001), fails to stimulate secretion in *Plumbago auriculata* and *Plumbago indica* (Fig 4). It has long been known that Na^+ and NH_4^+ stimulate secretion in *Drosera* tentacles (Darwin, 1893) so these ions were tested on the two species of *Plumbago* and *Drosera capensis* as well. Both NaCl and NH_4Cl stimulate secretion of protease at 5.0 mM concentrations in *Plumbago auriculata* (Fig. 3). KCl has less of an effect (Fig. 3) at the same concentrations. The 5 mM NaCl and NH_4Cl both stimulate secretion of protease by *Plumbago indica* as well. The secretion of protease increased as the concentration of the stimulus was increased as was evidenced by the more complete digestion in response to each of the stimuli (Fig. 3). This was true for *Plumbago auriculata*, *Plumbago indica*, and also *Drosera capensis*.

Stimulation by captured insects also resulted in proteases being secreted but it is unclear whether the protease comes from the insect or from plant secretions.

Mechanical stimuli seemed to have no effect. Preliminary experiments with a vibrating brush showed that mechanical stimulation alone did not increase the secretion of proteases. It is possible that mechanical stimulation might enhance the response to chemical stimulation as it has been shown to do in *Drosera capensis* (Lloyd, 1942).

Anatomy of the trichome resembles *Drosophyllum* stalked glands but is not precisely the same (Figs. 4, 5). A thicker layer of gland cells forms a cap above an endodermis similar to *Drosophyllum*. The stalk has an epidermis, a outer layer cortical parenchyma 3-5 cells thick and an inner core of narrow elongated parenchyma cells 7-12 layers thick (Figs. 4,5). The stalk is far thicker than that of *Drosophyllum* but lacks its pronounced vascular-ization. In at least some trichomes there appears to be a single vessel element entering the stalk but further investigation of the consistency of this feature is needed. The secretion of unstimulated trichomes stains positive with Sudan IV and negative with PAS indicating a lipid rather than a muscilaginous secretion like that found in *Drosophyllum*.

Discussion

Both *Plumbago auriculata* and *Plumbago indica* are capable of capturing insects and

secreting proteases in response to stimuli that would be expected to be present on the surface of insects and in decomposing insects. The absorption and assimilation of the material from captured prey has not been demonstrated and will be the subject of future study.

It is probable that *Plumbago*, like *Stylidium*, is carnivorous during flowering. This is a period when plants have a maximum demand for minerals since nutrients are being drained from the parent plant by seed production. It is likely that this type of carnivorous adaptation is more wide spread. Lloyd (Suda *et al.*, 2002) divided secretion on the glands of plants that catch small insects into three groups: oily (often aromatic), resinous, and mucilaginous. He states that carnivorous plants with adhesive traps all have mucilaginous secretions. It stands to reason that digestive and absorptive processes should more readily occur in an aqueous solution. Rachmilevitz and Joel (1976) reported that glands on *Plumbago* sepals have a resinous secretion. The secretion of unstimulated glands stains positive with Sudan IV and, therefore, must have a substantial hydrophobic constituent. Experiments testing the chemistry of stimulated glands are planned in the future.

References

Albert, V.A., Williams, S.E. and Chase, M.W., 1992. Science, **257**: 1497-1495.

Williams, S.E., Albert, V.A. and Chase, M.W., 1994. Am. Journ. Bot. **81**: 1027-1037.

Lledo, M.D., Crespo, M.B., Cameron, K.M., Fay, M.F. and Chase, M.W., 1998. Syst. Bot. **23**: 21-29.

Meimberg, H., Dittrich, P., Bringmann, G., Schlauer, J. and Heubl, G., 1999. Plt. Biol. **2**: 218-228.

Rashmilevitz, T. and Joel, D. M., 1976. Isr. Jour. Bot. **25**: 127-139.

Hartmeyer, S., 1997. CPN **26**: 39-45.

Carroll, D.B. and Darnowski, D., 2001. Unpublished manuscript.

Forssmann, W.G., Weche, I.S., Asoki, E., Drym, A. and Fawcett, D. W. 1977. Anat. Rec. **188**: 307-314.

Kelley, R.W., Dekker, A.F. and Bluemink, J.G., 1973. J. Ultrstr. Res. **45**: 254-258.

Darwin, C., 1893. Insectivorous Plants. Ams Press Inc., New York.

Lloyd, F., 1942. Carnivorous plants. Chronica Botanica Company, Waltham, MA.

Suda, J., Stoltzfus, A. and Williams, S. 2002. Jour. PA. Acad. Sci. **75**: 124.

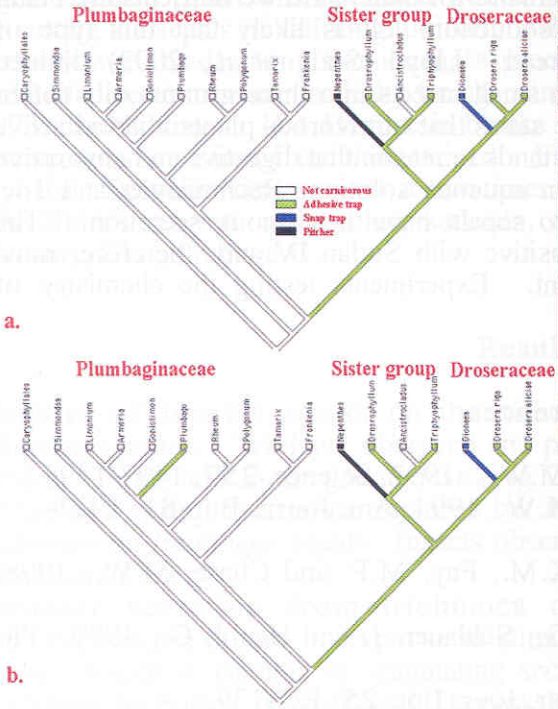

Fig 1. a) A phylogeny of Droseraceae, and related genera based on a cladistic analysis of the mat K gene (4). Carnivorous caryophyllids form a form a single clade Plumbago is related but not in the carnivorous clade

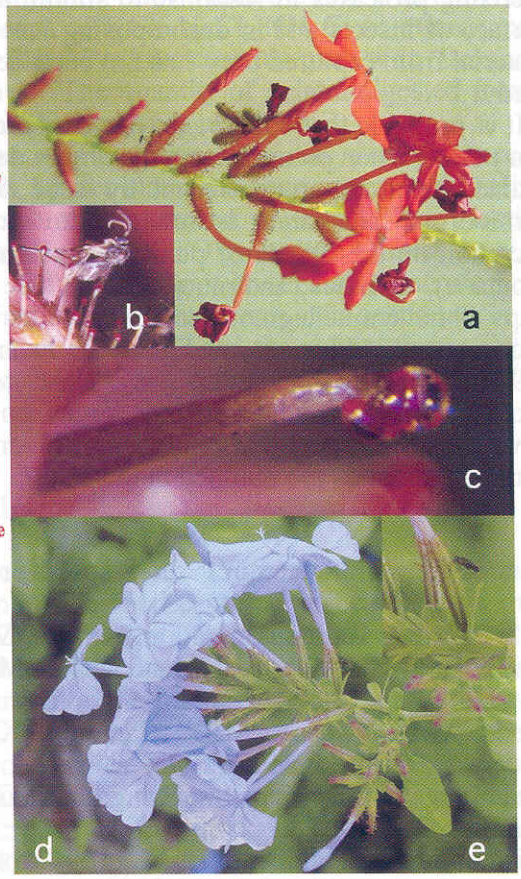

Fig. 2. a,b,c *Plumbago indica*; a) Inflor-escence, b) A captured fungus gnat, c) Trichome (150X). d,e *P. auriculata*; d) Inflorescence e) a captured spider.

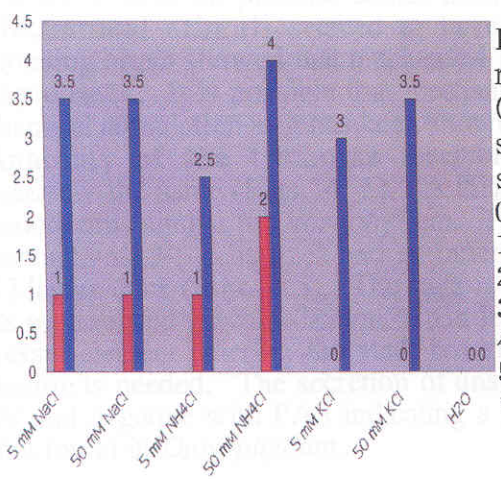

Fig. 3. A comparison of the relative responses of *Plumbago auriculata* (Blue) and *Plumbago indica* (Red) to chemical stimuli. Numbers show median gelatin substrate digestion after stimulation.
0 = no digestion
1 = pitting numbering < 20
2 = extensive pitting > 20 but no clear spots
3 = small clear holes ≤ 1 mm in diameter
4 = medium clear holes > 1 mm & < 5 mm in diameter
5 = large clear holes > 5 mm in diameter
* The values on the vertical scale are the median of six samples.

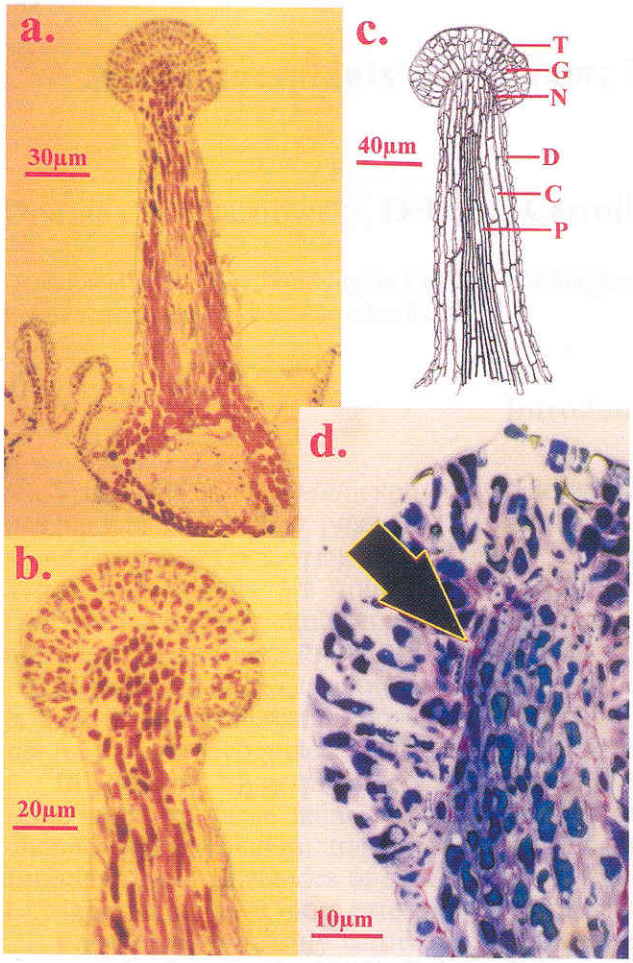

Fig. 4. *Plumbago auriculata*
a) (PAS) , b) l-section of trichome; c) Structures of trichome: G-gland cells, D-epidermis, T-cuticle, C-cortical parenchyma, P-elongated parenchyma, N-endodermis (also in "d" arrow points to casparian strip); d) (toluidine blue)

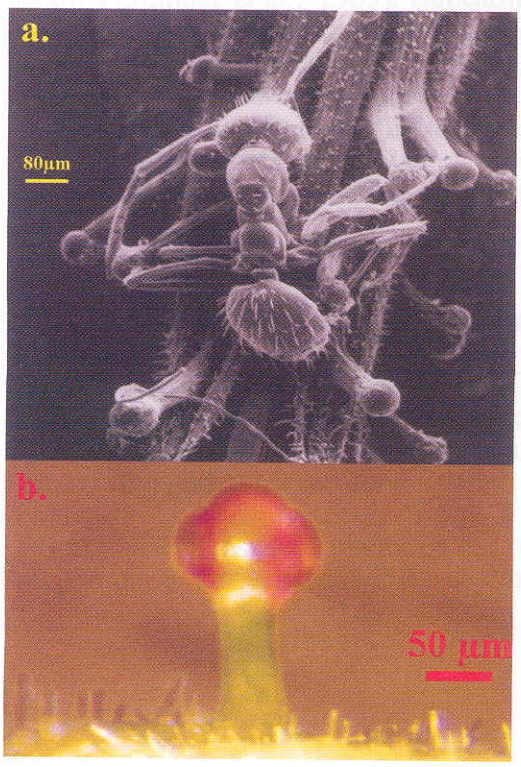

Fig. 5. *Plumbago auriculata*:
a) (SEM) of an ant captured on a sepal.
b) A live trichome illustrating the secretion droplet. The shape and angle of the stalked glands can be seen, as well as the hairs on the sepal.