# Effect of Storage on the Efficacy of Powdered Leaves of *Annona squamosa* for the Control of *Callosobruchus maculatus* on Cowpeas (*Vigna unguiculata*)

C.M.D. Dharmasena,[1,2] W.M. Blaney[1] and M.S.J. Simmonds*,[3]

*Annona squamosa* L. is widespread in Sri Lanka and its leaves are collected by some farmers to protect their stored cowpeas from predation by bruchids. Removal of the leaves during the dry season, when the farmers harvest their cowpeas, can decrease the yield of *A. squamosa* fruit. This study investigated whether storing the leaves before they are used would reduce the insecticidal activity of the leaves. Overall, acetone and ethanol extracts made from fresh and stored leaves of *A. squamosa* decreased the number of adult *Callosobruchus maculatus* (F.) emerging from cowpeas; however, in some bioassays the extracts from fresh leaves were more active than those from leaves stored for 6 months. Acetone extracts from fresh and stored leaves were toxic to adult beetles, whereas the ethanol extracts were not active. Acetone and ethanol extracts from fresh leaves had potent ovicidal activity when applied to 2-day-old eggs.

KEY WORDS: *Callosobruchus maculatus*; *Annona squamosa*; insecticide; botanicals.

## INTRODUCTION

The custard apple, *Annona squamosa* L., is grown as a fruit crop in many tropical countries and has been shown to have insecticidal properties (5,7). Ethanol, methanol and ether extracts of seeds and leaves of *A. squamosa* have been reported to be effective against insect pests of seeds such as *Callosobruchus chinensis* (L.) (6). The powdered leaves of *Annona reticulata* L., a species related to *A. squamosa*, were effective in decreasing the damage to green gram by the pulse beetle, *Callosobruchus maculatus* (F.) (8). Crude ethanol extracts of *A. squamosa* have also been shown to be effective in controlling the stored product pest *Tribolium castaneum* (Herbst) (11) and could be used to control human lice (10). The insecticidal properties of species of *Annona* were initially attributed to alkaloids, but more recent studies have demonstrated that a group of C-32 or C-34 linear fatty acids called acetogenins is associated with the insecticidal activity (7).

A survey of small-scale farmers in Sri Lanka showed that they frequently used leaves and extracts made from leaves of their *A. squamosa* trees to control pests of stored products, especially *C. maculatus* (2). In Sri Lanka farmers harvest their cowpeas in the dry season and they must treat the seeds before they are stored. Removing fresh leaves from *A. squamosa* during the dry season to make the extracts would reduce photosynthesis, remove

Received Oct. 30, 2000; received in final form Feb. 15, 2001; http://www.phytoparasitica.org posting May 6, 2001.
[1] Birkbeck College, University of London, London WC1E 7HX, UK.
[2] Present address: Rice Research and Development Institute, Batalagoda, Ibbagamuwa, Sri Lanka.
[3] Jodrell Laboratory, Royal Botanic Gardens, Kew, Richmond, Surrey TW9 3AB, UK. *Author for correspondence [Fax: +44-181-3325340; e-mail: m.simmonds@rbgkew.org.uk].

buds and, hence, reduce the yield of custard apple fruits. However, removal of the leaves in the wet season does not result in such a decrease in fruit. Before carrying out field trials with extracts made from *A. squamosa* leaves harvested at different times of the year, a series of laboratory experiments were undertaken. These experiments were designed to determine whether extracts from dried, stored leaves of *A. squamosa* were as effective in controlling *C. maculatus* on cowpeas as extracts from fresh leaves.

## MATERIALS AND METHODS

**Preparation of the plant material** Leaves of *A. squamosa* were collected from trees growing at the Royal Botanic Gardens, Kew, Surrey, UK. Extracts from these Kew-grown plants have been reported to control *C. maculatus* and had activity similar to extracts obtained from leaves of *A. squamosa* growing in Sri Lanka (2). The leaves from the Kew-grown plants were freeze-dried, ground in a mechanical grinder and sieved using a 200-mesh sieve (pore size 0.075 mm). Part of the sieved material was extracted in acetone and ethanol; the rest was stored in polythene bags at 25±1°C for 6 months before extraction. To make the acetone extract, the milled leaves (25 g) were placed in glass containers with acetone (250 ml) and left to extract for 3 days, after which the mixture was filtered using a Büchner funnel. Acetone in the filtrate was removed by evaporation under nitrogen. Ethanol extracts of *A. squamosa* leaves were prepared in a similar manner, except that a rotary evaporator was used to remove ethanol from the filtrate. The extracts from the fresh and stored leaves used in the experiments were made by dissolving a known weight of each filtrate in the respective solvent, acetone or ethanol. The extracts were tested within 5 days of being made.

**Insects** *C. maculatus* was cultured on seeds of *Vigna unguiculata* (L.) cv. 'California 6'. The culture was maintained at 30±1°C and 70% r.h. and the experiments were conducted under the same environmental conditions.

*Bioassays*

**Effect of extracts on adult mortality** To assess the efficacy of the extracts as insecticides, the bruchid adults were confined in glass vials (height 7.5 cm; diam 2.5 cm), the inner sides of which had been coated with an extract. This was achieved by adding an aliquot (1 ml) of an extract of *A. squamosa* to the vial, which was rolled slowly on its side to create a thin film of extract on its inner surface. Three concentrations (4%, 2% and 1% w/v) of each extract were tested, as well as controls. Three controls were tested: an acetone and ethanol solvent control, as well as solvent-free clean vials. The vials were left for 24 h and then five pairs of 48-h-old bruchids were introduced into each vial. The vials were closed with plastic stoppers, each with a gauze window for ventilation. The numbers of dead bruchids in each vial were counted after 24 and 72 h.

**Effect of extracts as surface protectants of cowpeas against oviposition** Sets of 25 cowpea seeds were placed in glass vials, as above, and 0.2 ml of one of the three concentrations (4%, 2% and 1% w/v) of acetone or ethanol extracts was pipetted onto the seeds in each vial. Each vial was shaken gently for 45 sec to ensure the seeds were coated with the extract, and then the seeds were placed on a filter paper for 24 h to allow the solvent to evaporate. Each set of seeds was divided into five groups of five seeds and each group was placed in a fresh vial with three 2- or 3-day-old adult bruchids (two females and one male) and the vials were closed, as described above. The bruchids were removed after 3 days and the white and translucent eggs on the seeds were counted after 12 days. Undeveloped eggs remain translucent, whereas viable eggs become white due to the frass produced by the neonate larva as it bores into the seed. Thus, the total number of white and translucent eggs on the seeds indicates bruchid oviposition and the number of white eggs indicates the number of larvae entering the seed. The number of adults subsequently emerging from seeds was noted on alternate days, starting 24 days after the parent bruchids had been placed in the vial. In order to avoid confusion with the second generation of adults, the adult count was stopped after 40 days.

**Effect of extracts on eggs already laid on cowpea seeds** Two hundred cowpea seeds and a colony of 100 bruchids were placed together in a container (20 cm diam) for 2 h. The bruchids were removed and seeds bearing four or five eggs were selected for the experiments. Twenty-five such seeds were placed in a glass vial and 0.2 ml of an acetone or ethanol extract at the concentration of 2% w/v was pipetted onto the seeds, either 2 or 6 days after the eggs had been laid. Each vial was shaken gently for 45 sec and the seeds were then placed on a filter paper for 24 h for the solvent to evaporate. The seeds were divided into groups of five and each group was placed in a vial as described above. Solvent-only and untreated controls were set up. The numbers of eggs and adults were counted, as described above. Embryonic development at 30 ± 1°C takes about 4–5 days, and the larvae can be seen within the egg after the 5th day; therefore, the 2- and 6-day post-oviposition treatments evaluate the ovicidal and larvicidal activities of the extracts, respectively.

**Statistics** The Mann-Whitney U test was used to: *(a)* compare the effect of treatments against their respective controls and *(b)* determine whether the activity of extracts from fresh leaves differed from that of extracts made from stored leaves.

## RESULTS

**Effect of extracts on adult mortality** Acetone extracts from both fresh and stored leaves caused adult mortality, which increased along with the duration of exposure to the extracts (Table 1). The highest concentration (4% w/v) of the acetone extracts from fresh and from stored leaves was insecticidal. The acetone extract from fresh leaves retained its activity at 1% w/v, whereas the extract from stored leaves lost its activity at this concentration. Overall, the ethanol extracts caused less adult mortality than the acetone extracts, and only the 4% w/v ethanol extract of stored leaves caused significant adult mortality after 72 h. However, there was no difference in the activity of extracts from fresh and stored leaves (Table 1).

**Effect of extracts as surface protectants of cowpeas against oviposition** Acetone extracts from fresh leaves decreased the number of eggs laid *by C. maculatus* on seeds treated with extracts at 4% and 2% w/v, whereas none of the acetone extracts from stored leaves deterred oviposition (Table 2). Only the highest concentration (4% w/v) of the ethanol extract from both the fresh and stored leaves deterred oviposition. Overall, the numbers of larvae entering the seeds and of adults emerging were decreased more by the acetone extracts of fresh leaves than by the ethanol extracts of fresh leaves, but the age of the leaves used to make the extracts did not influence the activity (Table 2). The acetone extracts of fresh leaves decreased the number of adults emerging from the seeds by 55% to 87%, relative to the number of adults emerging from untreated seeds; the 2% acetone extract from fresh leaves was more active than that from stored leaves. The 4% and 2% acetone extracts from stored leaves significantly decreased adult emergence. The 4% and 2% ethanol extracts from fresh leaves decreased the number of adults that emerged from 89% to 61% of the untreated control, whereas only the 4% extract from stored leaves decreased the number of adults that emerged. In all cases the number of adults that emerged decreased as the concentration of extract increased from 1% to 4% w/v.

TABLE 1. Effect of extracts of *Annona squamosa* on the mortality (number of dead out or ten) of adult *Callosobruchus maculatus*

| Concentration of extract (% w/v) | Mortality (mean ± SEM)[z] | | | |
|---|---|---|---|---|
| | Fresh leaves | | Stored leaves | |
| | 24 h | 72 h | 24 h | 72 h |
| | | *Acetone* | | |
| 4 | 2.4±0.91[y] | 8.9±0.49[y] | 3.2±0.84[y] | 8.6± 0.21[y] |
| 2 | 1.2±1.21[y] | 5.4±1.41[y] | 0.6±0.21[x] | 3.8±0.42[y] |
| 1 | 0.8±0.46 | 3.1±1.41[y] | 0.8±0.41 | 1.8±0.73[x] |
| Acetone control | 0.2±0.11 | 1.9±0.54 | 0.8±0.52 | 1.2±0.42 |
| Untreated control | 0.1±0.21 | 0.9±0.74 | 0.4±0.21 | 1.6±0.24 |
| | | *Ethanol* | | |
| 4 | 1.0±0.81 | 1.9±0.99 | 1.6±0.45 | 2.2±0.84[y] |
| 2 | 1.1±0.71 | 1.4±0.71 | 0.6±0.41 | 1.2±0.72 |
| 1 | 0.7±0.46 | 1.1±0.81 | 0.8±0.61 | 1.0±0.23 |
| Ethanol control | 0.2±0.15 | 0.4±0.24 | 0.4±0.72 | 1.0±0.72 |
| Untreated control | 0.1±0.52 | 0.5±0.54 | 0.4±0.71 | 0.6±0.24 |

n = five vials with ten insects per vial (five pairs).
[z] Number of dead insects per vial.
[y] Number of dead bruchids significantly greater than the untreated control, $P<0.05$.
[x] Extract from fresh leaves killed significantly more bruchids than the respective extract from stored leaves, $P<0.05$ (Mann-Whitney U test).

TABLE 2. Effect of extracts of *Annona squamosa* on oviposition, larval mortality and adult emergence of *Callosobruchus maculatus*

| Concentration of extract (% w/v) | Number per seed (mean±SEM) | | | | | |
|---|---|---|---|---|---|---|
| | Fresh leaves | | | Stored leaves | | |
| | eggs | larvae | adults | eggs | larvae | adults |
| | | | *Acetone* | | | |
| 4 | 7±1.8[z] | 7±1.8[z] | 0.4±0.14[z] | 17±1.9[y] | 10±1.2[z] | 0.6±0.14[z] |
| 2 | 6±1.5[z] | 6±1.4[z] | 0.8±0.41[z] | 17±1.5[y] | 13±1.2[z] | 2.2±0.71[z,y] |
| 1 | 20±1.8 | 15±1.8 | 1.4±0.68[z] | 22±0.8 | 19±1.5 | 2.6±0.35 |
| Acetone control | 17±2.4 | 14±1.9 | 2.6±1.54 | 18±0.7 | 16±0.6 | 2.6±0.24 |
| Untreated control | 19±2.4 | 16±1.6 | 3.1±1.23 | 16±2.1 | 14±2.1 | 4.0±0.51 |
| | | | *Ethanol* | | | |
| 4 | 10±2.6[z] | 8±1.4[z] | 0.4±0.25[z] | 11±0.1[z] | 8±0.2[z] | 1.9±0.51[z] |
| 2 | 13±1.8 | 10±3.7 | 1.4±0.47[z] | 13±1.9 | 12±1.7 | 2.6±0.62 |
| 1 | 14±3.8 | 11±3.7 | 3.4±0.98 | 13±0.6 | 12±0.5 | 3.4±0.52 |
| Ethanol control | 16±3.7 | 16±2.8 | 3.4±1.23 | 16±1.6 | 16±1.4 | 4.5±0.74 |
| Untreated control | 17±3.5 | 14±3.7 | 3.6±1.82 | 16±2.1 | 14±2.2 | 4.0±0.91 |

n = five vials with five seeds per vial.
[z] Number of eggs, larvae or adults per seed significantly less than the untreated control, $P<0.05$.
[y] Seeds treated with extract from fresh leaves had significantly fewer eggs, larvae or adults than seeds treated with extract from stored leaves, $P<0.05$ (Mann-Whitney U test).

**Effect of extracts on eggs already laid on cowpea seeds** The potency and mode of action varied among extracts. For example, the acetone and ethanol extracts of *A. squamosa* were effective in preventing the development of eggs in 2-day-old eggs, but not of larvae in 6-day-old eggs (Table 3). This indicates that the extracts had ovicidal activity. The results also show that the acetone extract from fresh leaves was more active than that from stored leaves in decreasing the number of larvae entering the seed. However, overall extracts from both fresh and stored leaves when applied to seeds with eggs decreased the number of emerging adults.

TABLE 3. Effect of 2% w/v acetone and ethanol extracts of *Annona squamosa* on development of *Callosobruchus maculatus* when applied to eggs at different times (2 or 6 days) after egg-laying

| Treatment and time after egg laying | Number per seed (mean±SEM) | | | |
|---|---|---|---|---|
| | Fresh leaves | | Stored leaves | |
| | larvae | adults | larvae | adults |
| | | *Acetone* | | |
| 2 days, extract | 0.4±0.12[z] | 0.4±0.46[z] | 1.3± 0.18[z,y] | 0.2±0.12[z] |
| 2 days, control | 2.6±1.24 | 1.4±0.75 | 2.5±0.22 | 1.4±0.05 |
| 6 days, extract | 1.9±0.74 | 0.2±0.06[z] | 3.5±0.47 | 0.1±0.04[z] |
| 6 days, control | 2.9±0.65 | 1.1±0.23 | 3.6± 0.21 | 0.5±0.22 |
| Untreated | 3.6±0.34 | 1.4±0.39 | 3.4±0.27 | 1.5±0.22 |
| | | *Ethanol* | | |
| 2 days, extract | 0.4±0.25[z] | 0.4±0.17[z] | 1.2±0.31[z] | 0.2±0.19[z] |
| 2 days, control | 2.8±0.29 | 1.1±0.57 | 2.5±0.23 | 1.4±0.45 |
| 6 days, extract | 2.4±0.57 | 0.4±0.16[z] | 3.5±0.55 | 0.1±0.05[z] |
| 6 days, control | 3.4±0.69 | 1.4±0.35 | 2.7±0.32 | 0.7±0.16 |
| Untreated | 2.9±0.27 | 1.4±0.74 | 3.4±0.24 | 1.5±0.01 |

n = five vials with five seeds per vial.

[z] Number of eggs, larvae or adults per seed significantly less than the untreated control, $P<0.05$.

[y] Seeds treated with extract from fresh leaves had significantly fewer eggs, larvae or adults than seeds treated with extract from stored leaves, $P<0.05$ (Mann-Whitney U test).

## DISCUSSION

Over 120 plants and plant produce have been shown to have insecticidal or deterrent activity against stored product pests (1). Currently many farmers in parts of Africa and Asia use some of these botanicals to protect their legumes from attack by bruchids, with varying degrees of success (3,4,9). However, the number and quality of plants used by farmers is often limited by their availability (2). *A. squamosa* is an evergreen tree in Sri Lanka and its leaves are potentially available for extracting by farmers for bruchid control throughout the year. However, Sri Lankan farmers harvest their cowpeas in January, the Maha season, and in July, the Yala season. In January and July the rainfall is low and it is not advisable to prune a fruit crop such as *A. squamosa* during the dry seasons. It would be better to prune the tree in November and in May, when there is sufficient rainfall to promote new shoot growth. This study has shown that extracts made from leaves dried, ground and stored for 6 months can decrease the number of bruchids emerging from treated cowpeas. Therefore, it might be possible for farmers to prune the trees during the wet seasons and then dry the leaves prior to use when they harvest their cowpeas.

In Sri Lanka the farm-gate price of cowpeas is high in April, during the Sinhala-Hindu new year period (2). Therefore, it is financially beneficial for farmers to ensure that their cowpea crop is protected and in good condition for this festival. Our results have shown that if farmers were able to apply extracts made from stored *A. squamosa* leaves, they could provide some protection to their harvested seeds by decreasing the buildup of bruchid populations in the seeds.

Storage of leaves of *A. squamosa* did not cause an overall decrease in the efficacy of the organic extracts in decreasing the emergence of adults emerging from treated cowpeas. Nevertheless, there were some differences in insecticidal activity that could be associated with a decrease in the levels of active compounds in the extracts made from stored leaves. However, no chemical analysis has yet been undertaken on these extracts to determine whether the differences in activity could be attributed to qualitative and/or quantitative differences in the profile of compounds in these extracts.

Many studies (1) report the potential use of botanicals without reference to their toxicity to non-target organisms. A full risk analysis needs to be carried out on extracts of *A. squamosa* before a low-cost pest control strategy using *A. squamosa* can be devised. Such an analysis should take into account the effects of the extracts on seed viability as well as on natural predators, parasitoids and public health. To date, work undertaken in the USA has shown that the acetogenins, the compounds thought to be responsible for the insecticidal activity of *A. squamosa*, are not likely to be mutagenic (7). However, further work needs to be undertaken in Sri Lanka to transfer these laboratory-based experiments to field trials in which the inherent variability in bruchid behavior and plant chemistry on the viability of *A. squamosa* extracts can be assessed, as well as the feasibility and cost of making organic extracts.


## ACKNOWLEDGMENTS

The first author thanks the Department of Agriculture and the Agricultural Research Project of Sri Lanka for financial assistance to conduct these experiments as part of his Ph.D. research. We thank E. Porter and M. Cullum for technical support, and the anonymous reviewers for their constructive comments on the manuscript.


## REFERENCES


1. Dale, M.J. (1996) A Review of Plant Material Used for Controlling Insect Pests of Stored Products. NRI Bull. 65, Natural Resources Institute, Chatham, UK.
2. Dharmasena, C.M.D. (1995) The control of *Callosobuchus maculatus* (F.) in cowpeas in Sri Lanka: varietal resistance, conventional insecticides and locally available botanicals. Ph.D. thesis, University of London, UK.
3. Dharmasena, C.M.D., Simmonds, M.S.J. and Blaney, W.M. (1998) Insecticidal activity of eight plant species on egg laying, larval development and adult emergence of *Callosobruchus maculatus* (F.) in cowpea. *Trop. Agric. Res. Ext.* 1:67-69.
4. Don-Pedro, K.N. (1990) Insecticidal activity of fatty acid constituents of fixed vegetable oils against *Callosobruchus maculatus* (F.) on cowpea. *Pestic. Sci.* 30:295-302.
5. Grainge, M. and Ahmed, S. (1988) Handbook of Plants with Pest-Control Properties. John Wiley & Sons, New York, NY.
6. Islam, B.N. (1987) Use of some extracts from Meliaceae and Annonaceae for control of rice hispa, *Dicladispa armigera*, and the pulse beetle, *Callosobruchus chinensis*. *in*: Schmutterer, H. and Ascher, K.R.S. [Eds.] Natural Pesticides from the Neem Tree and Other Tropical Plants. Proc. Third Int. Neem Conf. (Nairobi, Kenya, 1986), pp. 217-242. Eschborn, Germany.
7. McLaughlin, J.L., Zeng, L., Oberlies, N.H., Alfonso, D., Johnson, H.A. and Cummings, B.A. (1997) Annonaceous acetogenins as new natural pesticides: recent progress. *in*: Hedin, P.A., Hollingworth, R.M., Masler, E.P., Mijamoto, J. and Thompson, D.G. [Eds.] Phytochemicals for Pest Control. *ACS Symp. Ser.* 658:117-133.
8. Rajapaksa, R.H.S. (1990) Effect of five botanicals as protectants of greengram against the pulse beetle *Callosobruchus maculatus*. *in*: Fujii, K., Gatehouse, A.M.R., Johnson, C.D., Mitchell, R. and Yoshida, Y. [Eds.] Bruchids and Legumes: Economics, Ecology and Coevolution. pp. 85-90. Kluwer Academic Publishers, Dordrecht, the Netherlands.
9. Singh, S.R. [Ed.] (1990) Insect Pests of Tropical Food Legumes. Wiley, Chichester, UK.
10. Sokvanichislip, N., Gritsanapan, W., Somanabandhu, A., Lekcharoen, K. and Tiankrop, P. (1994) Toxicity testing of organic solvent extracts from *Annona squamosa*: Effects on rabbit eye and ear skin. *Phytother. Res.* 8:365-368.
11. Williams, L.A.D. and Mansingh, A. (1993) Pesticidal potential of tropical plants. 1. Insecticidal activity of leaf extracts of sixty plants. *Insect Sci. Appl.* 14:697-700.