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The *in vitro* Culture of Rose-Gall Tissue Induced by the Cynipid Wasp *Diplolepis spinosa* (Ashmead)

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Abstract

Galls induced on *Rosa rugosa* Thumb. by the cynipid wasp *Diplolepis spinosa* (Ashmead) were surface sterilized and cut open. The layer of plant tissue that lines the gall chamber was excised and was an excellent source of tissue for initiating *in vitro* callus cultures. The interior of the gall chamber proved to be sterile and there is evidence that anti-microbial compounds may be produced by young gall tissue.

Keywords: Rose, galls, wasps, cynipid

1. Introduction

Colony forming wasps in the genus *Vespa* are well known but there are other smaller members of the order Hymenoptera such as the cynipid and chalcid wasps. These are around 2–4 mm long and are associated with plants. In Canada, *Rosa rugosa* Thumb. and its hybrids are hosts to both chalcid seed wasps and gall-inducing cynipid wasps (Shorthouse, 1994; Nalepa and Grissel, 1993; Gillan and Richardson, 1997). *Rosa rugosa* is widely planted, being winter-hardy and having scented flowers followed by fleshy orange hips (Verrier, 1991).

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Chalcid wasps lay their eggs in the young achenes (seeds) within the hips and the larvae consume the embryo including the cotyledons, thereby reducing the reproductive capacity of the plant (Nalepa, 1991). The cynipid wasps of the genus *Diplolepis* have an even more complex association with their rose host. They induce the rose to form galls. The females lay their eggs in the vegetative tissues of the rose and the larvae induce a gall, the shape and size of which is unique for the species of wasp within (Shorthouse, 1993a).

The nutritional interactions between gall-inducing insects and their plant hosts are beginning to be understood (Shorthouse and Rohfritish, 1992). About 30 species of cynipid wasps are found on wild roses in North America (Shorthouse, 1993a,b) and three attack *Rosa rugosa* in Canada. Two of these wasps, *Diplolepis spinosa* (Ashmead) and *Diplolepis radicum* (Osten Sacken) are quite widespread (Shorthouse, 1988); the first species inducing galls on the stems and the second on the roots. The third species, *D. fulgens* (Gillette) which is less common is also a root-gall inducer (Shorthouse, 1994).

Diplolepis spinosa, investigated in the present study, induces spherical galls, up to the size of a golf ball, on the stems of *R. rugosa* (Fig. 1a). Following the gall-initiation phase, and during the growth phase, the chamber in which the insect larva lies is lined with nutritive cells containing a rich supply of nutrients (Fig. 1b). The larvae slice these cells open and suck up the exuding nutrients (Shorthouse, 1993b). These cells superficially resemble callus cells that develop when plant parts (explants) are cultured *in vitro*. Callus cells have been used to initiate *in vitro* suspension cultures or for plant regeneration using tissue culture techniques. This led us to investigate whether it might be possible to culture nutritive cells from rose galls and to investigate whether antimicrobial compounds were produced within the gall.

2. Materials and Methods

Diplolepis spinosa galls were obtained during the summers of 1993 and 1994 from *Rosa rugosa* plants on the south side of the Student Centre Building on the campus of Saint Mary's University, Halifax, Nova Scotia. Galls were also collected in late June, 1995, from Sudbury, Ontario and in mid-August, 1995, from the Saint Mary's site.

The galls were surface sterilized for 10–15 minutes in 95% ethanol and rinsed in sterile distilled water. They were then cut open on a laminar air-flow bench and the outer hard layers removed with scalpel and forceps. The inner pale yellow tissue was cut into pieces from 2 to 20 mm in diameter and transferred to *in vitro* growth media. The medium used was developed by Arnold et al. (1992) and was a three quarter strength Murashige and Skoog salts medium with NH_4NO_3 adjusted to 1856 ppm and full strength micronutrients with

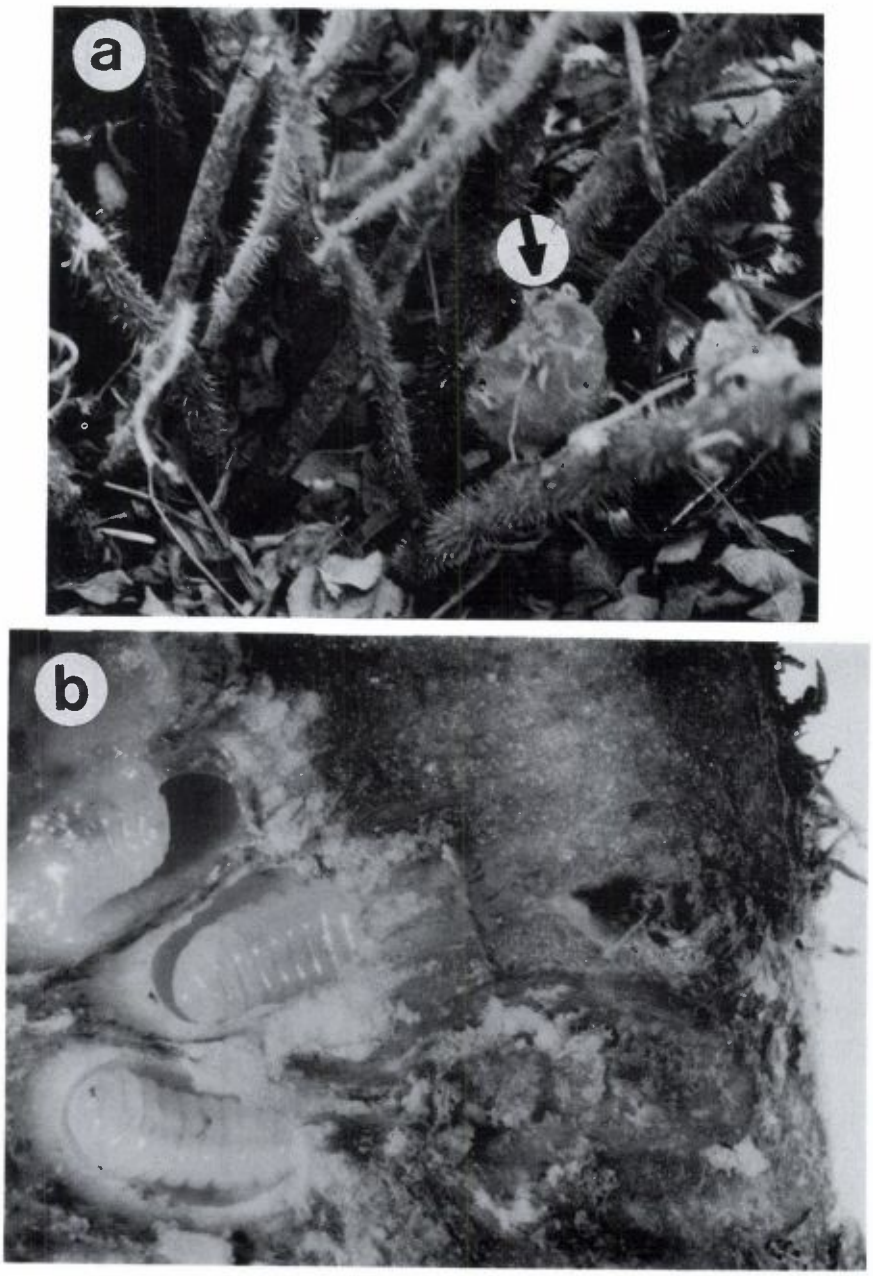

Figure 1. a) Bush of *Rosa rugosa* showing a gall (arrow) caused by *Dipolepis spinosa*; b) Section through a maturing gall showing the multichambered nature of the gall and the contained *Dipolepis spinosa* larvae.

$ZnSO_4.7H_2O$ and $MnSO_4.H_2O$ altered to 21.2 and 33.8 ppm, respectively. Sucrose was added at 3% and phytagel at 0.6%. The pH was adjusted to 5.7 before autoclaving at 121°C for 20 minutes. Media were prepared containing either 0.25 μM kinetin + 0.5 μM NAA, or a range of hormone combinations that included 0–80 μM 2,4-D and 0–10 μM kinetin. The cultures were kept in a growth room with a 16-hour photoperiod and a temperature of 26°C with a light intensity of 30–40 $\mu E\ m^{-2}\ s^{-1}$ and a 16-hour photoperiod.

The galls and stems, in a further study, were surface sterilized as described above. Then, plugs of gall tissue were removed from the inside using a surface sterilized 5 mm cork borer. The plugs were excised from the area of the gall containing the larval chambers (Fig. 1b). The cores were cut into 1 mm thick slices. Pieces of rose stem about 5 mm in diameter were also cut into transverse sections about 1 mm thick.

Discs of gall tissue and stem tissue were placed on petri dishes containing nutrient agar, the surface of which had been inoculated with a 24-hour broth culture of either *Escherichia coli* or *Staphylococcus aureus*. Erythromycin antibiotic discs were also placed on the bacterial plates. The plates were incubated for 24-hour at 37°C by which time the bacterial carpet was clearly visible. Any zones of inhibition were recorded and photographed.

3. Results

The immature galls, collected in June, were 1–2 cm in diameter, light green and easily sliced open with a scalpel. The mature galls collected in mid-August were 3–4 cm in diameter, hard with dark brown patches and were very difficult to cut open. Five to seven days after excision of gall tissue from around the larval chamber and transfer to *in vitro* culture media, callus was observed developing around the gall tissue. By this time the original gall tissue had darkened to pale brown (Fig. 2a).

For callus initiation, a medium containing kinetin and NAA was used, but faster growth occurred subsequently on media containing 2,4-D. Growth was optimal on 40 μM 2,4-D. Eight pieces of callus, with an average fresh weight of 1.08±0.06 mg, grew to a mean of 490±20 mg fresh weight after six weeks growth. After three months, variation was observed in the callus, some cultures being green and compact while others were friable and cream coloured. Rhizogenesis was quite common in the treatments with lower hormone levels. Reduced growth occurred at 80 μM 2,4-D and no growth occurred in the absence of plant hormones.

Discs of immature gall tissue induced zones of inhibition when placed on a petri plate containing either *E. coli* or *S. aureus* (Fig. 2b). These were seen as broad, clearly demarcated zones around each piece of gall tissue. The stem

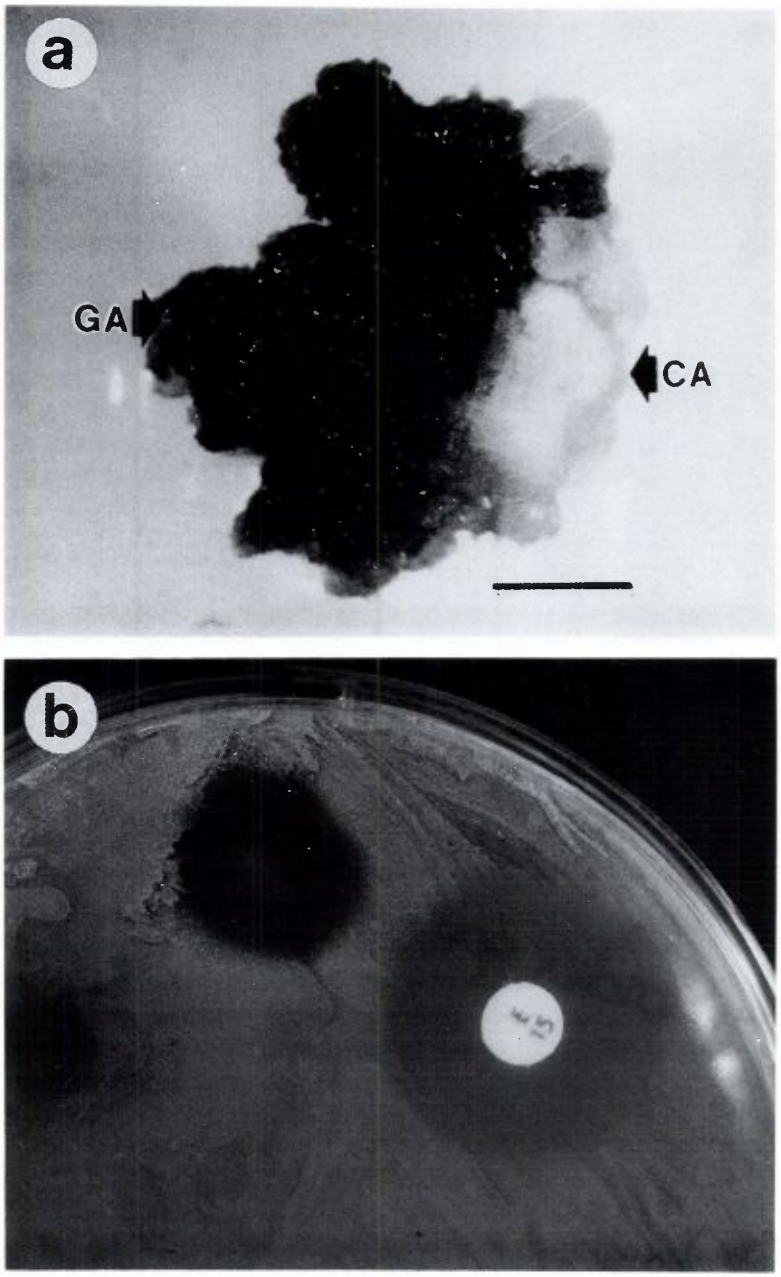

Figure 2. a) Callus tissue developed after five days of culture of an explant from the inner tissue of a rose gall. GA = gall tissue, Ca = callus, The scale bar is 0.25 cm; b) Petri plate which had been coated with *Escherichia coli* and on which a rose stem section (left), a disc of gall tissue (centre) and an erythromycin antibiotic disc (right) had been placed and then incubated for 24 hours.

pieces did not show clear inhibition zones and contamination quickly developed around them. Similar discs taken from mature gall tissue did now show comparable inhibition zones.

4. Discussion

Rohfritsch (1971) tried to get portions of cynipid galls induced on *Glechoma hederacea* L. by *Aulax glechomae* L. to continue growing *in vitro*. She found that presence of the larvae was necessary for the nutritive layer to continue to form and for differentiation of hard protective layers in the outer gall. In the present study, the gall tissue continued to grow in the absence of the larvae; the rose cells being stimulated by the plant hormones added to the media.

In roses, the larvae must produce substances that induce morphogenetic growth responses since the size and shape of gall induced by each *Diplolepis* species is characteristic. These substances have yet to be characterized. It is also clear that the larvae must induce multiplication readiness in the nutritive cells within the gall as they multiply so readily in in *vitro* culture.

Apical meristems or stem segments are used to initiate *in vitro* micro-propagation systems (e.g., Douglas et al., 1989; Arnold et al., 1992) but for genetic engineering, or growing cells in suspension culture for the production of medicinal compounds, callus cells are required. In the latter context, attempts have been made to genetically engineer roses and introduce novel genes, especially blue pigments into the flowers (Holten and Tanaka, 1994). Callus cells can be transformed rather easily and if plants can be regenerated, a novel variety carrying the new gene can be grown and distributed. As callus cells can be grown from the inner cells of insect-induced galls in a few days, there is obvious potential for using plant galls as a source of cells for molecular biology research.

Many plants are hosts of gall-inducing insects (Redfern and Askew, 1992). The observation, in the present study, that the interior of the gall chamber remains sterile was very interesting. The lack of contamination by bacteria or fungi was unexpected and reflects a sterile larval chamber. Presumably, this allows the thin walled nutritive cells to multiply and be progressively sliced open by the feeding larvae without the larva or the chamber being overgrown by saprotrophic micro-organisms. In this context it is significant that the larvae do not defecate while feeding (Shorthouse, 1993b).

The indication that gall tissue contains compounds that are antibiotic also raises the question as to which partner in these insect-plant relationships is responsible for the production of antibiotic and morphogenetic factors. How is interaction and control brought about in such symbioses? Unlike galls on roses induced by *Agrobacterium tumifaciens*, no transfer of genetic material such as a

plasmid seems to occur (Farkas and Haas, 1985; Poncet et al., 1995). In galls induced by the bacterium, the Ti plasmid induces the host cell to produce the plant hormones cytokinin and auxin so that cultures derived from such cells grow on media in the absence of added growth regulators. Cultures derived from rose gall tissue did not grow without such regulators.

The observations reported in this paper and a summary of previous studies on the morphological interactions and life histories of these gall wasps (Shorthouse and Rohfritsch, 1992) was presented the Second International Symbiosis Congress at Woods Hole. We drew attention to the topic of plant-insect relationships through a consideration of one host, *Rosa rugosa*. It is hoped that there will be more papers on gall-inducing insects at the next Congress because these symbioses involve complex fascinating interactions and are also of economic potential as biological control agents.

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REFERENCES

Arnold, N.P., Binns, M.R., Barthakur, N.N., and Cloutier, D.C. 1992. A study of the effect of growth regulators and time of plantlet harvest on the *in vitro* multiplication rate of hardy and hybrid tea roses. *Journal of Horticultural Science* **67**: 727–735.

Douglas, G.C., Rutledge, C.B., Casey, A.D., and Richardson, D.H.S. 1989. Micropropagation of floribunda, ground cover and miniature roses. *Plant Cell Tissue and Organ Culture* **19**: 55–64.

Farkas, E. and Haas, J.H. 1985. Biological control of crown gall in rose nursery stock. *Phytoparasitica* **13**: 121–127.

Gillan, T.L. and Richardson, D.H.S. 1997. The chalcid seed wasp, *Megastigmus nigrovariegatus* Hymenoptera:Torymidae, on *Rosa rugosa* Thumb. in Nova Scotia. *Canadian Entomologist* **130**: 809–814.

Holton, T.A. and Tanaka, Y. 1994. Blue roses – a pigment of our imagination. *TBTECH* **12**: 40–42.

Nalepa, C.A. 1991. Distribution of the rose seed chalcid *Megastigmus aculeatus* var. *nigroflavus* Hoffmeyer (Hymenoptera: Torymidae) in North Carolina. *Journal of the Entomological Society* **24**: 413–416.

Nalepa, C.A. and Grissell, E.E. 1993. Host seed size and adult size, emergence, and morphology of *Megastigmus aculeatus nigroflavus* (Hymenoptera: Torymidae) in North Carolina. *Journal of Entomological Science* **22**: 1313–1317.

Poncet, C., Antonini, C., Bettachini, A., Hericher, D., Pionnat, S., Simonini, L., Dessaux, Y., and Nesme, X. 1995. Impact of the crown gall disease on vigor and yield of rose trees. *Proceedings de la Congres Rosier*, pp. 1–2.

Redfern, M. and Askew, R.R. 1992. *Plant Galls*. Naturalists Handbooks, no. 17, Richmond Publishing, Slough.

Rohfritsch, O. 1971. Culture *in vitro* de jeunes galles d'*Aulax glechomae* L. sur *Glechoma hederacea* L. *Marcellia* **37**: 151–161.

Shorthouse, J.D. 1988. Occurrence of two gall wasps of the genus *Diplolepis* (Hymenoptera: Cynipidae) on the domestic shrub rose, *Rosa rugosa* Thumb. (Rosaceae). *Canadian Entomologist* **120**: 727–737.

Shorthouse, J.D. 1993a. Cynipid galls of shrub roses. *American Rose* (May issue), 16–17.

Shorthouse, J.D. 1993b. Adaptations of gall wasps of the genus *Diplolepis* (Hymenoptera-Cynipidae) and the role of gall anatomy in cynipid systematics. *Memoirs of the Entomological Society of Canada* **165**: 139–163.

Shorthouse, J.D. 1994. Host shift of the leaf galler *Diplolepis polita* (Hymenoptera: Cynipidae) to the domestic shrub rose *Rosa rugosa*. *Canadian Entomologist* **126**: 1499–1503.

Shorthouse, J.D. and Rohfritsch, O. 1992. *Biology of Insect-Induced Galls*. Oxford University Press, New York.

Verrier, S. 1991. *Rosa Rugosa*. Capability's Books, Deer Park.