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Developmental morphology of galls induced by *Diplolepis rosaefolii* (Hymenoptera: Cynipidae) on the leaves of *Rosa virginiana* and the influence of *Periclistus* species on the *D. rosaefolii* galls.

A thesis submitted to the graduate faculty

in partial fulfilment of the requirements

for the degree of

Master of Science

in the Department of Anatomy and Physiology

Faculty of Veterinary Medicine

University of Prince Edward Island

Deborra A. LeBlanc

Charlottetown, PEI, Canada

July, 1997

Chairman of the Department of Anatomy and Physiology

Faculty of Veterinary Medicine

University of Prince Edward Island

Charlottetown, P. E. I.

Canada C1A 4P3

SIGNATURE PAGES

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To my children, Christopher, Andrena, and Delia.

I hope that you too will come to know the joy of learning

and not see it as a burden imparted by others.

Galls of Diplolepis rosaefolii

on the leaflets of Rosa virginiana

Abstract

The larval stages of the cynipid wasp *Diplolepis rosaefolii* induce the formation of single-chambered, lenticular galls on the leaves of the wild shrub rose, *Rosa virginiana*. The gall consists of four tissue layers which surround a centrally located larval chamber. These include an outermost dermal layer, underlying parenchyma, sclerenchyma, and nutritive tissue. Development in the

D. rosaefolii galls involves a number of characteristics which are unique to this gall, and differ markedly from development in other *Diplolepis* galls studied. These characteristics include the presence of double sclerenchymal layers and the lack of vascularization in the gall wall. *Periclistus* is an inquiline in the galls induced by *D. rosaefolii*. Under the influence of *Periclistus* larvae, a number of morphological changes, including an increase in the number of larval chambers, were observed in the galls. Initially, a loss of *D. rosaefolii*-induced nutritive tissue is seen when *Periclistus* eggs are laid in the galls. Once the *Periclistus* larvae hatch, the number of cells which make up the parenchyma increases. Changes in tissue type, proportion, and overall morphology exhibited by *Periclistus*-modified galls were studied using conventional light microscopy techniques.

Acknowledgements

I would like to take this opportunity to thank all the people who have helped me throughout my Master's degree. Without your expertise, assistance, and support this research and thesis would not have been possible. Of these people, there are some to who I owe a special thank you:

- To my supervisors, Dr. Christian Lacroix and Dr. Glenda Wright, for their guidance, encouragement, and support. Thank you for allowing me to do my Master's research in a field of my own choosing, and for helping to make this an interesting and exciting experience.
- To the members of my supervisory committee for letting a botany/entomology student into their realm and treating her like one of their own. I even forgive you for not making it to the party!
- To the faculty and staff of the Department of Anatomy and Physiology for their assistance and support.
- To Shelly and Tom in photography for always coming through for me, no matter how late my requests were submitted.
- To Dorota Wadowska for her technical assistance and for allowing me the use of the microtomes.
- To Dr. Joe Shorthouse for identification of wasps and for participating in my degree as my external examiner.
- To my parents, Tom and Marlene Llewellyn, who believe in me and support me in all things.
- To my friends, Caroline Baglole and Larry Yeo, for supporting me and being there for me.
- To the Harvey Moore Wildlife Fund for scholarships received in both 1995 and 1996.
- To the Canadian Federation of University Women for a scholarship received in 1995.
- To UPEI for financial assistance through a Senate Research Grant awarded to Dr. Christian Lacroix.
- To NSERC for financial support through an NSERC operating grant (# OGP0121550) awarded to Dr. Christian Lacroix.

Table of Contents

Title page....i
Conditions of use of thesis....ii
Permission to use thesis....iii
Dedication....iv
Certificate of thesis work....iv
Frontispiece....vi
Abstract....vii
Acknowledgements....viii
Table of Contents....ix
List of Figures....xi
List of Tables....xv

1. Introduction....1
1.1 Insect galls....1
1.2 Biology of *Diplolepis*....2
1.3 Biology of *Diplolepis rosaefolii*....3
1.4 Cynipid Inquilines....3
1.4.1 *Periclistus*....5
1.5 Cells of the primary plant body in dicotyledons....6
1.5.1 Ground tissues....6
1.5.2 Complex tissues....7
1.6 *Rosa virginiana*: general morphology and early development....9
1.7 Cynipid galls: gross morphology....10
1.8 Gall development....12
1.9 Inquiline modified galls....13
1.10 Research objectives....14

2. Materials and Methods....15
2.1 Collection techniques....15
2.2 Tissue fixation and processing....16
2.3 Light microscopy....17
2.3.1 Spurr's resin....17
2.3.2 LR White resin....18
2.3.3 Sectioning and photography....18
2.4 Quantitative analysis....19
2.5 Scanning electron microscopy....20

3. Results **22**
3.1 *Rosa virginiana*: normal leaf histology 22
3.2 Galls of *Diplolepis rosaefolii* 22
3.2.1 External morphology of galls 22
3.2.2 External morphology of larvae 25
3.2.3 Developmental morphology of galls of *Diplolepis rosaefolii* 30
3.2.3.1 Stage 1: Initiation 30
3.2.3.2 Stage 2: Nutritive tissue formation 35
3.2.3.3 Stage 3: Sclerification 38
3.2.3.4 Stage 4: Maturation 41
3.2.4 Statistical analysis of *Diplolepis rosaefolii* galls 44
3.3 *Periclistus*-modified galls 48
3.3.1 External morphology of galls 48
3.3.2 External morphology of the larvae 48
3.3.3 Gall development 53
3.3.3.1 Egg deposition and initial changes in the galls 53
3.3.3.2 Further changes in gall tissues induced by *Periclistus* larvae 53
3.3.3.2.1 Nutritive tissue 53
3.3.3.2.2 Parenchymal tissue 56
3.3.3.2.3 Sclerenchymal tissue 59
3.3.3.2.4 Summary of changes in *Periclistus*-inhabited galls 60

4. Discussion **68**
4.1 Changes in leaf morphology 68
4.2 *Diplolepis rosaefolii* gall development 70
4.3 Changes in gall morphology induced by *Periclistus* 74
4.4 Summary 78

5. References **81**

List of Figures

1. Cross section of a mature leaflet of *Rosa virginiana.*

2. Newly initiated *Diplolepis rosaefolii* galls on young leaflets of *Rosa virginiana.*

3. Young galls of *Diplolepis rosaefolii* on *Rosa virginiana* leaflets.

4. Adaxial view of a gall prior to maturation.

5. Adaxial view of mature gall of *Diplolepis rosaefolii.*

6. Newly hatched *Diplolepis rosaefolii* larva.

7. Young *Diplolepis rosaefolii* larva.

8. Maturing *Diplolepis rosaefolii* larva.

9. Mature *Diplolepis rosaefolii* larva.

10. Light micrograph of *Diplolepis rosaefolii* eggs showing their location on the secondary veins of a leaflet.

11. Scanning electron micrograph of a stage similar to Fig. 10 showing pedicels of the eggs.

12. Scanning electron micrograph showing the site of egg attachment on the secondary vein.

13. Higher magnification of Fig. 12 showing the ovipositional fluid at the site of attachment of the egg to the leaflet.

14. Cross section of a leaflet showing the location of the point of attachment of an egg on the abaxial epidermal layer.

15. Higher magnification of a stage similar to that in Fig. 14 showing cellular changes in the vicinity of the point of attachment of the egg to the leaflet.

16. Low magnification of cross section of a folded leaflet blade showing the pad of tissue which forms on either side of the attachment site of the egg.

17. Detail of the site of attachment of the egg as depicted in Fig. 16 showing fragmented vacuolation of the adjacent cells.

18. Low magnification of the final phase in the initiation stage.

19. Detail of Fig. 18 showing the presence of lysed cells surrounding the chamber.

20. Cross section of a gall showing an early stage of nutritive tissue development restricted to one side of the larval chamber.

21. Higher magnification of Fig. 20 showing the occlusion of the pathway of entry of the larva.

22. Detail of the nutritive tissue showing the vacuolar gradient and variations in cell size from the larval chamber towards the periphery of the gall.

23. Cross section of undifferentiated leaf tissue from *Rosa virginiana* leaf bud showing the meristematic cells.

24. Cross section of a young gall in which the nutritive tissue surrounds the larval chamber.

25. Scanning electron micrograph of a cross section through a gall.

26. Early stage of sclerification highlighted by the presence of secondary wall thickenings in cells underlying the adaxial epidermis of the leaflet.

27. Later stage of sclerification showing primary sclerenchymal plates above and below the larval chamber.

28. High magnification of sclerenchyma cells showing the secondary wall and the ring of cytoplasm surrounding the large central vacuole.

29. Detail of nutritive tissue showing pronounced vacuolar gradient and variation in cell size from the larval chamber to the periphery of the gall.

30. Cross section through a gall chamber showing extension of the primary sclerenchymal plates to the periphery of the gall.

31. Higher magnification of Fig. 30 showing details of the nutritive tissue.

32. Early stage in maturation showing different tissue types.

33. Higher magnification of the adaxial gall wall in Fig.32.

34. Higher magnification of the abaxial gall wall in Fig. 32.

35. Higher magnification of the nutritive tissue found laterally in the gall from Fig. 32.

36. Detail of the lateral sclerenchyma present in the gall wall at later stages of maturation.

37. Mature abaxial gall wall.

38. Mean cell diameters for tissue types in *Diplolepis rosaefolii* galls at various stages of development.

39. Galls containing *Diplolepis rosaefolii* and *Periclistus* larvae.

40. Young *Periclistus*-inhabited gall, showing lightly pigmented central area on the exposed portion of the gall.

41. Darkly pigmented, mature galls inhbited by *Periclistus* are illustrated .

42. Newly hatched *Periclistus* larva.

43. Maturing *Periclistus* larva showing the mandibles and well-defined body segments.

44. Mature larvae of *Periclistus* showing the mandibles and a series of three ridges stretching across the dorsal surface of each segment.

45. Dorsal view of *Diplolepis rosaefolii* gall chamber showing *Periclistus* eggs.

46. Cross section of *Diplolepis rosaefolii* gall, showing enclosed *Periclistus* egg.

47. Cross section of the abaxial gall wall showing attachment of *Periclistus* egg.

48. Higher magnification of Fig. 47 showing details of the attachment site of a *Periclistus* egg inside the gall chamber.

49. Cross section of young *Periclistus*-inhabited gall .

50. Detail of nutritive tissue of gall shown in Fig. 49.

51. Cross section of a maturing *Periclistus*-inhabited gall.

52. Cross section of mature *Periclistus*-inhabited gall.

53. Detail of thin-walled and highly vacuolated parenchyma cells lateral to the gall chamber in *Periclistus*-inhabited gall.

54. Cross section of the adaxial wall of a *Periclistus*-inhabited gall showing the patches of parenchyma cells underlying the epidermal layer.

55. Cross section of *Periclistus*-inhabited gall chamber with a single sclerenchymal plate above and below the gall chamber.

56. Detail of an adaxial gall wall in a *Periclistus*-inhabited gall, showing undulations in the upper sclerenchymal plate isolating areas of chloroplast-containing parenchyma tissue.

57. Cross section of a *Periclistus*-inhabited gall with double sclerenchymal plates above and below the larval chamber.

58. Cross section of an adaxial gall wall in a *Periclistus*-inhabited gall showing undulations of the secondary sclerenchymal layer and the heavy deposition of secondary cell wall in the cells of the primary sclerenchymal layer.

59. Cross section of abaxial gall wall in a *Periclistus*-inhabited gall with single sclerenchyma layer surrounding a vascular bundle.

60. Detail of abaxial gall wall in a *Periclistus*-inhabited gall with double sclerenchyma layer.

61. Initial stage of sclerenchyma differentiation in *Periclistus*-inhabited gall showing secondary wall thickenings in cells adjacent to the larval chamber.

62. Mature lateral sclerenchyma in *Periclistus*-inhabited gall.

63. Higher magnification of lateral sclerenchyma shown in Fig. 62.

List of Tables

1. Summary of developmental changes in tissue layers of *Diplolepis rosaefolii* galls.

2. Analysis of mean cell diameters in various tissue layers of *Diplolepis rosaefolii* galls.

3. Summary of major developmental changes in galls inhabited by *Diplolepis rosaefolii* and *Periclistus* larvae.

1. Introduction

1.1 Insect galls

Insect galls are atypical growths induced in plants. They arise as the result of an interspecific association between a plant and an insect (Rohfritsch 1992). Gall morphology is influenced by two genotypes: that of the insect, which provides the stimulus, and that of the plant, which determines the growth response (Weis and Abrahamson 1986). Cynipid wasps (Hymenoptera; Cynipidae) induce highly differentiated, morphologically complex galls with up to five distinct tissue layers. These include: i) nutritive tissue, ii) vascular tissue, iii) cortical parenchyma, iv) sclerenchyma, and v) epidermal tissue (Mani 1964; Cornell 1983; Meyer and Maresquelle 1983). Gall inducers are unique in that they actively manipulate the host plant through mechanical and/or chemical stimuli to form a structure which provides the inducer with both nutrition and shelter (Shorthouse 1993; Askew 1984).

A large percentage of galls are host to a number of other wasps which secondarily inhabit the structures (Narendran 1984; Schönrogge *et al.* 1994). These secondary inhabitants are: i) inquilines, which are phytophagous and feed only on the gall tissue, and ii) parasitoids, which feed on any larvae they find in the gall. Inquiline species have the ability to modify the galls they inhabit, although they cannot induce gall formation (Shorthouse 1975).

1.2 Biology of *Diplolepis*

Cynipid wasps of the genus *Diplolepis* Geoffroy (formerly the genus *Rhodites* Hartig) are widely distributed, with most species being found in North America (Shorthouse and Ritchie 1984). All species restricted to North America induce galls on rose (*Rosa*) species. Galls induced by cynipid wasps may be found on the leaves, stems, buds, or roots of various rose species. The site of induction is specific to the wasp species. Many wasps have the potential to induce galls on more than one species of rose (Stille 1984). With few exceptions, the galls have a characteristic shape and structure, which are unique to the species of gall-inducer.

All *Diplolepis* species are univoltine (i.e. produce one set of offspring per year or season) and they do not exhibit alternation of generations (i.e. produce two sets of offspring a year which are morphologically distinct from each other) as seen in many other species of cynipids (Askew 1984; Rohfritsch 1992; Shorthouse 1993). Many cynipid wasps are parthenogenic (offspring from unfertilized eggs) and therefore males are not common (Kinsey 1920). However, Callan (1940) reared males of *D. rosae* from unfertilized eggs, and Stille and Davring (1980) found that these males are reproductively inactive. Stille and Davring (1980) postulate that the genus is obligate homozygous automictic deuterotoky, a type of parthenogenesis in which unfertilized eggs go through meiotic division, followed by the fusion of two nuclei to give rise to both male and female offspring (Chapman 1971). Since all larvae are found in individual chambers, *Diplolepis* galls can be referred to as monothalmous (single chambered) or polythalmous (multi-chambered).

Most species of *Diplolepis* exhibit a similar life cycle (Shorthouse 1975, 1993). In the spring, adults exit the galls and search for a suitable developing plant structure on which to oviposit. Adults live for three to five days (Kinsey 1920). The emergence of adults is synchronized with the availability of plant tissues suitable for gall initiation. Most *Diplolepis* species exit their galls in the early spring when the first buds are forming on the host plant. Some cynipid wasps have the ability to revert differentiated tissue back to a meristematic state when the larva exerts its influence on the plant (Rohfritsch 1992). Eggs of some species are attached to a single epidermal cell (Bronner 1985; Rohfritsch 1992). The eggs have a lytic effect on underlying tissue resulting in the formation of a chamber which the larvae enter after they hatch (Bronner 1977). The initiation of gall tissues does not occur until the larvae begin to feed. Larvae overwinter in the prepupal stage inside the gall. In early spring the larvae pupate and adults exit by chewing through the gall wall (Shorthouse 1975; 1993).

Diplolepis-induced galls grow very rapidly and evidence of gall formation can be detected as early as two days after the larvae hatch (Shorthouse 1975; Rohfritsch 1992). Larvae feed on the nutritive cells that line the gall chamber. However, the larvae do not ingest the whole cells, but tear into the nutritive cells with their mandibles and suck out the contents. As the contents are consumed by the larvae, the adjoining cells develop cytological features similar to the consumed cells (such as increased numbers of organelles, and enlarged nucleus and nucleolus), and then they too are eventually consumed (Bronner 1992; Rohfritsch 1992). At gall maturity, the chamber is lined with thick-walled sclerenchyma cells and collapsed nutritive cells (Meyer and Maresquelle

1983). Larvae are fully grown after approximately two months (Shorthouse 1975). The time for completion of larval development depends on a number of factors, including condition of the host plant and time of oviposition (Shorthouse 1975).

1.3 Biology of *Diplolepis rosaefolii*

Diplolepis rosaefolii (Cockerell) was described by Cockerell (1889) from galls collected in Colorado, U.S.A. Galls induced by *D. rosaefolii* are found on rosebushes in central and eastern North America (Beutenmuller 1907). The larvae of *D. rosaefolii* induce the formation of lenticular-shaped galls on the leaves of *Rosa* spp. Initial studies have shown that the mature galls range in size from 3.0 to 6.0 mm in diameter and are found protruding from both the adaxial (upper) and abaxial (lower) leaflet surfaces (LeBlanc 1995). The galls are monothalmous and are usually found singly or in dense clusters on individual leaflets. Most mature galls are predominantly red and easily distinguished on the leaves.

1.4 Cynipid Inquilines

Many inquilines have a close taxonomic relationship to the inducer (Shorthouse 1973). Mani (1964) postulated that inquilines were originally gall inducers who secondarily began to feed on the galls of other inducers. Ronquist (1994) has proposed the term agstoparasites to describe parasites such as cynipid inquilines that are closely related to their host species (e.g. species in the same family or genus). He believes that cynipid inquilines evolved monophyletically as a single line from one cynipid host and

subsequently moved onto other hosts. In contrast, Shorthouse (1980) suggested that inquilines may be evolving and their ability to induce galls is still limited to modify the galls induced by other insects. Askew (1984) listed six inquilines associated with cynipid galls which belong to the family Cynipidae. They include *Periclistus*, *Ceroptres*, *Euceroptres*, *Saphonecrus*, *Synophromorpha*, and *Synergus* species.

1.4.1 Periclistus

Periclistus species are inquilines in *Diplolepis* galls (Shorthouse 1975). All *Periclistus* females associated with *Diplolepis* galls kill the *Diplolepis* larva with their ovipositor when they oviposit into the gall (Shorthouse 1980). Eggs are deposited on the inside surface of the larval chamber and once hatched the larvae begin to feed on the gall tissue (Shorthouse 1973). As feeding continues, the cells of the inner wall of the gall grow to surround each larva in its own individual chamber. *Periclistus* larvae overwinter in the gall and pupate in the spring. The adults exit from the gall, mate, and then search for immature galls in which to oviposit. Emergence of adults is synchronized with the presence of immature galls of the host wasp species (Shorthouse 1975).

Although *Periclistus* species do not induce galls, they modify the structures of galls induced by other cynipids (Shorthouse 1980). *Periclistus* larvae cause the formation of individual chambers around themselves, thereby allowing several larvae to occupy the same gall. In many *Diplolepis* galls this results in galls which are much larger than those containing only the larva of an inducer.

1.5 Cells of the primary plant body in dicotyledons

The primary plant body of dicotyledons consists of ground, dermal, and vascular tissues (Mauseth 1988). The ground tissue system includes simple tissues such as parenchyma, collenchyma, and sclerenchyma. The epidermis and the vascular tissues (xylem and phloem) make up the complex tissues.

1.5.1 Ground tissues

Classification of plant cells in the ground tissue is based on the nature of the cell walls. Parenchyma cells have thin primary walls, collenchyma consists of thick primary walls, and sclerenchyma has both primary and secondary walls (Mauseth 1988). Primary cell walls are composed of cellulose, hemicelluloses, pectin, and glycoprotein (Raven *et al.* 1986). The pectin provides plasticity which allows the walls to stretch during elongation of the organs in the plant. The primary cell walls contain thin areas which are called primary pit-fields. The cytoplasmic connections between adjacent cells, known as plasmodesmata, are often found in the pit fields. Secondary cell walls also contain cellulose and hemicellulose (Raven *et al.* 1986). Because pectins and glycoproteins are not present, the amounts of cellulose and hemicellulose are much greater in the secondary wall than in the primary wall. The lack of pectin and the increased cellulose provides greater rigidity and less plasticity to the secondary wall. The secondary wall is not laid down over the primary pit-fields and therefore pits are still present after secondary wall formation (Raven *et al.* 1986).

Parenchyma cells, found predominantly in continuous masses throughout the

plant body, are living at maturity, and are capable of dividing (Mauseth 1988). Parenchyma cells are highly vacuolated and contain plant organelles including chloroplasts and mitochondria.

Collenchyma cells are living at maturity and have unevenly thickened primary walls. They function in providing elastic support to the stem and shoot tip of the plant. Collenchyma cells are found mainly under the epidermis and around the vascular bundles in leaves and stems (Esau 1977).

Sclerenchyma cells have thick, lignified secondary cell walls and are usually dead at maturity. These secondary cell walls are very rigid. Sclerenchyma cells function in strengthening and supporting the plant body, and are usually found in the cortex of stems, although they are also associated with the phloem in the vascular tissue (Esau 1977).

1.5.2 Complex tissues

The epidermis is the outermost layer of cells in the primary plant body (Raven *et al.* 1986). It is usually one cell thick and provides some mechanical support to the plant because of its compact arrangement. External walls of epidermal cells in aerial parts of the plant are covered with a cuticle, a layer of cutin and wax that helps prevent water loss (Esau 1977). Most cells in the epidermis are parenchymal. However, a number of specialized cells are also present including guard cells and trichomes. Guard cells are chloroplast-containing cells in the aerial portions of the plant which regulate the opening and closing of the stomata (pores between the guard cells), thereby controlling the

movement of gases in and out of the plant (Salisbury and Ross 1992). Trichomes are outgrowths of individual epidermal cells. They include hairs, scales, and water vesicles. Trichomes carry out a number of functions including defence against insects, regulating of leaf temperatures through creation of a boundary layer, and facilitating in absorption of water and minerals (Esau 1977).

The vascular tissues consist of xylem and phloem (Esau 1977). Xylem is the water conducting tissue of the plant. There are two types of conducting cells present in the xylem of angiosperms: i) tracheids and ii) vessel elements (Salisbury and Ross 1992). Both cell types are elongated and are dead at maturity. They contain primary and secondary walls which may contain pits. The end walls and sometimes the lateral walls of the vessel elements contain perforated plates, which are areas without primary or secondary walls and hence form holes in the cell wall (Salisbury and Ross 1992). Vessel members join end-to-end, forming long tubes called vessels. Tracheids do not have perforated plates. However the pits in their cell walls are concentrated at the end of the cells. Xylem also contains parenchyma which functions in storage (Raven *et al.* 1986).

Phloem is the principal food conducting tissue of the plant (Salisbury and Ross 1992). The conducting cells are called sieve elements. The conducting cells found in higher plants are called sieve-tube members and they occur in longitudinal strands called sieve tubes. The end walls of sieve-tube members have sieve plates which contain large pores (Esau 1977). The sieve-tube members are alive at maturity but they lack nuclei, ribosomes, and Golgi bodies. The organelles which are present in the sieve-tube members are distributed along the cell wall. The sieve-tube members contain a primary wall only.

The phloem tissue also contains parenchyma (Salisbury and Ross 1992). A specialized group of parenchyma cells called companion cells are present in the phloem and are normally associated with the sieve-tube members. They contain all organelles in their protoplast, including many mitochondria. Large numbers of ribosomes are also present in the protoplast (Salisbury and Ross 1992). There are numerous plasmodesmatal connections between sieve-tube elements and companion cells. It is believed that companion cells provide metabolites to the sieve-tube elements. Sclerenchyma cells may also be present in the phloem and are found associated with the sieve-tube elements (Esau 1977).

1.6 *Rosa virginiana*: gross morphology and early development

Rosa virginiana (Mill) (Rosaceae) is a sturdy, highly branched shrub with a stout stem and few suckers. The shrubs grow one to two meters high and are common throughout Prince Edward Island, growing along roadsides, in wet pastures, and along the heads of saltmarshes, dykelands, and swamps (Erskine 1985, Roland and Smith 1985). The pinnately compound leaf of *R. virginiana* is composed of a pair of basal stipules and two to four pairs of opposite leaflets born laterally on the rachis. There are five to seven leaflets which are oblong to oval (Gleason 1952). The leaflets are coarsely toothed, with teeth averaging 1 mm in height. The stipules partially surround the stem and enclose the single axillary bud. The flowers are usually solitary and found on branches from old wood, rather than on the one-year stems. The flowers have five petals which are 2-3 cm long (Gleason 1952).

Early development of *R. virginiana* leaves occurs on the shoot apex (LeBlanc unpublished data). The shoot apex is cylindrical and flattened apically, giving it a drum-shaped appearance. Leaf primordia are initiated at the periphery of the apex and first appear as swellings which become flattened on the adaxial side to form a dorsiventral, peg-like structure (LeBlanc unpublished data). Development of the leaf to this stage occurs in an acropetal direction. However, once leaflet formation begins, development becomes basipetal (LeBlanc unpublished data).

1.7 Cynipid galls: gross morphology

Cynipid galls consist of several tissues which are arranged in concentric, well-defined zones and may include up to five tissue layers (Mani 1964; Cornell 1983; Meyer and Maresquelle 1983). These tissue layers include nutritive tissue, parenchyma, sclerenchyma, vascular tissue, and epidermal tissue. The larval chamber (or chambers if it is a multi-chambered gall) in the centre of the gall is lined with nutritive cells that are rich in proteins, sugars, lipids, and RNA (Bronner 1992; Rohfritsch 1992). These nutritive cells are characterized by an abundance of cytoplasm containing numerous organelles (including ribosomes, plastids, and mitochondria), enlarged nuclei and nucleoli, and fragmented vacuoles (Shorthouse 1986; Bronner 1992). Peripheral to the nutritive layer is the sclerenchymal layer. This multicellular layer has been called the "protective layer" because it is thought to hamper parasitoid attack (Cornell 1983). However, many galls are heavily attacked by parasitoids and therefore the role of the sclerenchyma as a protective layer is questionable (Shorthouse 1975; Hawkins 1988). Sclerenchyma may also serve a

supportive role in the gall structure. Beyond the sclerenchymal layer there is a multicellular layer of cortical parenchymal cells (Shorthouse 1975). These are bounded by the epidermal cells which make up the outermost layer of the modified plant structure. Shorthouse (1975) used paraffin sections to study the morphology of six *Diplolepis* species [*polita* (Ashmead), *bicolor* (Harris), *lens* (Weld), *nebulosa* (Bassett), *ignota* (Osten Sacken), and *gracilis* (Ashmead)] that induce leaf galls on three species of *Rosa* in western Canada. The author found that each species induced a structurally distinct gall and that all the galls were made up of distinct tissue layers arranged concentrically around the centrally located larval chamber. Hough (1952) studied spangle galls of *Neuroterus quercus-baccarum* L. on the leaves of *Quercus* and found that the tissue layers of the gall were arranged above and below the larval chamber or to the side of the chamber. Even though galls of cynipid wasps are highly differentiated and their morphology is widely reported (Hough 1952; Shorthouse 1975; Bronner 1977), most research on the development of galls has been restricted to inducer-inhabited galls.

Few studies in gall morphology have utilized the unique perspective obtained through scanning electron microscope (SEM). The images of specimens produced by the SEM compliment and extend the information which is obtained using light microscopy. Specimens prepared for SEM can remain relatively intact and therefore the surface topography as well as any exposed internal features of the specimen can be observed (Goldstein *et al.* 1992). Anthony *et al.* (1983), and Anthony and Sattler (1990) used SEM to observe surface features of galls induced by the mites *Aceria fraxinivora* (Nal.) and *Eriophyes cladophthirus* (Nal.) on the leaves of *Fraxinus ornus* L. (ash) and *Solanum*

lycopersicum L. (tomato) respectively, but internal morphology was not observed in this manner.

1.8 Gall development

The development of most cynipid galls that have been investigated can be divided into three phases: initiation, growth, and maturation (Rohfritsch 1992). The overlap between the phases, and the duration of each phase vary with the species of the inducer, the species of the host plant, and the season when the galls are induced. Galls induced in the spring tend to grow faster than galls induced in the autumn, and gall formation may require weeks or months to complete (Rohfritsch 1992).

Initiation begins with oviposition of eggs on or within a specific organ of the host plant (Mani 1964; Shorthouse 1975). It is during the initiation phase that the newly hatched larva exerts control over the development of the plant cells in its vicinity, resulting in changes in the level of physiological activity of the affected plant cells and in their growth patterns. Cosens (1912) and Mani (1964) believe that the substance which causes this response originates in the salivary secretions of the larva, and may be a heterauxin (plant growth promoting hormone) or acts like one. However, Rohfritsch and Shorthouse (1982) propose that the larvae exert their influence on the growth pattern of the plant by mechanical means through the frequency and orientation of their feeding.

The growth phase of the gall involves an increase in cellular mass of the plant tissue surrounding the larva and this increase is achieved through hyperplasia and hypertrophy (Cosens 1912; Shorthouse 1975). Hyperplasia involves cell proliferation and

sometimes occurs without cytokinesis resulting in a polyploid condition in gall cells. Hypertrophy is an increase in cellular mass without division. Hypertrophy continues to the end of the growth phase and may persist after hyperplasia has ceased. During the growth phase, cells lining the larval chamber differentiate into nutritive cells which are very important both in feeding the larva and in the morphogenetic development of the gall (Rohfritsch 1992). The orientation of the larva while feeding is responsible for the direction of growth of the cells in the gall and causes the definitive shape of the gall (Rohfritsch and Shorthouse 1982).

The maturation phase marks the end of gall growth and the beginning of the active feeding stage of the larva (Lalonde and Shorthouse 1984). Differentiation of plant cells occurs, causing the development of several tissue layers, such as sclerenchyma, arranged concentrically around the larval chamber.

1.9 Inquiline modified galls

The enclosed larvae and the highly nutritious tissues of cynipid-induced galls attract other wasp species which secondarily inhabit the galls. These include inquilines which are phytophagous and feed only on the gall tissues, and parasites which are entomophagous and feed on the enclosed larvae (Shorthouse 1973). Bronner (1981) examined the developmental anatomy of inquiline-modified galls of *Neuroterus quercus-baccar* L. on *Quercus pendunculata* Erhl. and *Pediaspis aceris* (Gmelin) on *Acer pesudoplatanus* L. using semi-thin resin sections (0.65μm). She found changes in the morphology, cytology, and cytochemistry of the galls when the inquilines took over the

gall development. These changes included increased vacuolation in nutritive cells and changes in the location of starch and lipids within the cells of the gall wall in inquiline-modified galls. Shorthouse (1980) used paraffin sections (8 μm) to study modifications of *D. polita* galls on the leaves of *Rosa acicularis* when inhabited by the inquiline *Periclistus pirata* (Osten Sacken). He found morphological and cytological changes in the tissues of the galls, including changes in the size and cellular contents of the nutritive cells and changes in the arrangement of cells in the sclerenchymal layer. An increase in the size of the galls was also noted. To this date, no work has been done on the morphology of *D. rosaefolii* galls, nor on the inquiline-modified galls of this species.

1.10 Research objectives

The purpose of this study was i) to investigate the developmental morphology of galls induced by *Diplolepis rosaefolii* on *Rosa virginiana* using both high resolution light microscopy (resin-embedded tissue sectioning) and scanning electron microscopy; and ii) to investigate the changes in morphology which occur in *D. rosaefolii* galls when the inquiline *Periclistus* sp. inhabits the gall using high resolution light microscopy. It is hypothesized that a) significant changes in leaf tissues will take place as larvae of *D. rosaefolii* inhabit the leaf, b) growth of the *D. rosaefolii* gall will occur through hypertrophy in all tissue layers, and c) further changes in the tissue layers and general morphology of the *D. rosaefolii* galls will occur as inquilines colonize the galls.

2. Materials and Methods

2.1 Collection techniques

Galls were collected biweekly in 1995 (July to September: 12 weeks) and 1996 (June to October: 14 weeks) from *Rosa virginiana* bushes in Brackley Beach, Prince Edward Island (63°12'12"W, 46°25'20"N). Leaflets with eggs on them were found by careful examination of newly opened leaves of *R. virginiana* in areas of the rosebush where galls had been found previously. Leaflets with eggs have a pinkish tint when observed at close range. Confirmation of egg presence on the leaflets was carried out in the laboratory using a stereoscope. Random samples of galls were hand-collected by examining each section of the rosebush and taking the first galled leaf encountered. Each sample contained approximately 150 to 200 galls, and a total of 3500 galls were collected over the two year study period. Leaves picked from the rosebushes were placed in Whirl-pak® bags and transported to the laboratory. Leaves were removed individually from the bags and each gall was cut from the leaf tissue with a razor blade. Following removal from the leaf, newly initiated galls were placed directly in glutaraldehyde, while older galls were first dissected under a stereoscope to determine the inhabitants present. Newly initiated galls were those which were barely visible to the naked eye. Only galls that contained larvae of *D. rosaefolii* or *Periclistus sp.* were used in further processing. Initially, the approximate stages of gall development were determined by visual observation of size changes in the gall diameter. The sections were used to study the development of the various tissue layers, and to determine the developmental series. The

developmental stages were based on changes in the tissues and are not synonymous with the phases referred to in some gall papers (Shorthouse 1975, Rohfritsch 1992).

Ten leaflets (collected during July and August, 1996) and fifteen winter buds (collected in November 1996) of *R. virginiana* were collected and processed for serial sectioning following the same procedures as outlined for the galls. Leaves and stems of *R. virginiana* were collected and sent to Carol-Ann Lacroix at the Herbarium, University of Guelph, Guelph, Ontario for identification, and the identification was confirmed. Adults of both wasps were sent to Dr. J. D. Shorthouse, Laurentian University, Sudbury, Canada for identification. Once identification of the wasps was confirmed by Dr. Shorthouse, type specimens of adult *D. rosaefolii* and *Periclistus sp.* were deposited in the insect collection at the Eastern Cereal and Oilseed Research Centre, Agriculture and Agri-food Canada, Ottawa, Canada.

2.2 Tissue fixation and processing

All tissue fixation and dehydrations were carried out at room temperature following the procedures of Hayat (1981) and O'Brien and McCully (1981). Chemicals used for processing of all samples for light and scanning electron microscopy were purchased from J.B. EM Service Inc. (Montreal, Canada), unless otherwise stated. Gall tissues were fixed in 5% glutaraldehyde in a 0.1M phosphate buffer (pH 7.2) for 12 hours. Following primary fixation, samples were rinsed three times in 0.1 M phosphate buffer (pH 7.2) for 30-minute intervals. Galls from samples were randomly assigned for embedding in either Spurr resin or LR White resin. Samples to be embedded in Spurr

resin were post fixed in 2% osmium tetroxide in 0.2 M phosphate buffer (pH 7.2) for two hours, and rinsed three times in 0.2 M phosphate buffer (pH 7.2) for three 30-minute intervals. Samples that were to be embedded in LR White resin were post fixed in 1% phosphotungstic acid in 0.1M phosphate buffer (pH 7.2) for four hours, and rinsed three times in 0.1M phosphate buffer (pH 7.2) for 30 minutes.

2.3 Light microscopy

Following fixation, gall samples were dehydrated in a graded ethanol series from 25% to 100%, with 20-minute intervals in 25% and 50%, one hour in 75%, 30 minutes in 90%, and three 30-minute intervals in 100%.

2.3.1 Spurr's resin

Samples were embedded following the recipe for a medium mixture (Spurr 1969). Alcohol-dehydrated tissues were infiltrated for one hour in a mixture of two parts ethanol to one part Spurr resin, then put through two changes in a 1:1 mixture of ethanol and Spurr resin for one hour each, and finally in 100% Spurr resin for one hour. All infiltration was carried out on a rotator (TAAB vari-speed with 1 in. vial chambers, J.B. EM Service Inc. Montreal, Canada). Samples were then embedded in a fresh mixture of Spurr resin in labelled flat moulds (J.B. EM Service Inc., Montreal, Canada) and polymerized overnight at 70°C. Gall samples were oriented with the long axis of the leaf perpendicular to the block face.

2.3.2 L R White resin

Samples were embedded following the procedure of Newman (1987). Dehydrated samples were infiltrated for one hour in a mixture of two parts ethanol to one part L R White resin, then put through two changes in a 1:1 mixture of ethanol and L. R. White resin for one hour each, and finally in 100% L. R. White resin overnight. Sections were embedded in a fresh mixture of L. R. White resin in labelled gelatin capsules (size 0: 7mm diameter, 0.68 ml, Marivac Ltd, Halifax, Canada) and polymerized overnight at 60°C. Gall samples were oriented with the long axis of the leaf perpendicular to the block face. Although sections from galls embedded in L. R. White were better than sections from galls embedded in Spurr's resin, the difference in the two sections was not enough to justify the additional time required to process and remount L. R. White blocks. Therefore, only sections from blocks embedded in Spurr's resin were used for photography.

2.3.3 Sectioning and photography

Serial sections of approximately 0.30 μm thick, were cut using a Reichert-Jung Ultracut or Porter-Blum MT2-B ultramicrotome. At least 10 galls at each stage of development were sectioned. All stains used were purchased from Fisher Scientific Co., Nepean, Canada. Serial sections were mounted on glass slides (Fisher-brand precleaned plain microscope slides, Fisher Scientific Co., Nepean, Canada) and stained with 1% toluidine blue in 1% sodium borate (after Kramer and Windrum 1955). Additional

sections were mounted on slides and contrasted with other types of stains to enhance or target specific cellular inclusions. These stains included 1% methylene blue in 1% sodium borate (general plant stain; after Sheehan and Hrapchak 1980), methylene blue-azure A–basic fuschin (lignified walls, cellulose walls, and cuticle; after Humphrey and Pitman 1974), azure II–methylene blue (lignified walls; after Richardson *et al.* 1960), Sudan black B (simultaneous staining of starch and lipids; after Bronner 1975), and safranin and fast green (cellulose walls and lignified walls; after Conn 1953). Sections stained with Sudan Black B revealed starches and lipids in the cells, but a dark precipitate was present on all sections which could not be removed, even after repeated rinsing. Therefore, photographs of these sections were not included in this thesis. Photographs of all other sections were taken on an Olympus BH2 microscope fitted with a SC35 camera using Kodak Tmax film and printed on Kodak polycontrast RC III paper.

2.4 Quantitative analysis

Digital images of gall sections were captured using Semicaps® software. Measurements of parenchymal, sclerenchyma, and nutritive cells were taken at all stages of development. For each stage of development, 10 measurements of each cell type were obtained using a balanced, randomized design from four randomly selected galls. Measurements were taken from sections close to the midpoint of the gall chamber and each cell was measured at its widest diameter using Semicaps® software. Statistical analyses included: i) mean diameters with standard deviations for cells at each stage of development and ii) analysis of variance of the differences between stages for each cell

type. All statistical analyses were done using Minitab® (1994).

2.5 Scanning electron microscopy

Following fixation as described above, 25 galls and 20 leaflets were dehydrated in a graded ethanol series from 25% to 70%, with 20-minute intervals in 25% and 50%, and overnight in 70%. Leaflets to be scanned for eggs on the surface were placed in two overnight changes in 100% ethanol. The remainder of the galls were put through a graded tertiary butyl alcohol (TBA) series from 5% to 75%. Galls were left in each step overnight to ensure proper dehydration. Samples were further dehydrated by two overnight changes in 100% TBA, one at 37°C and another at 61°C. Specimens were then transferred to a 50-50 solution of TBA and paraffin at 61°C and left overnight, and were then subjected to two changes in 100% paraffin at 61°C overnight.

Specimens were subsequently embedded in paraffin and cut with a rotary microtome (American Optical model 820 microtome, American Optical Co., Buffalo, New York) until the gall chamber was reached following the procedures of Kemp *et al.* (1993). Specimens were trimmed to remove excess paraffin and then placed in xylene to solubilize the paraffin. Galls were transferred to fresh xylene and left until all the paraffin was removed.

All galls were critical point dried in a Ladd 28000 critical point drier using CO_2 as the transitional fluid. Dried specimens were mounted on aluminum stubs with two-sided adhesive tape, and silver paint was applied to the base of the specimens to ground them. Specimens were coated with gold palladium in a Denton Vacuum Desk II sputter coater

to a thickness of 30 nm. All specimens were viewed with a Cambridge Stereoscan 604 scanning electron microscope. Digital images were captured using Semicaps ® software and printed on thermal paper using a Mitsubishi P67U video copy processor.

3. Results

3.1 *Rosa virginiana*: normal leaf histology

The epidermal cells of the leaf (Fig. 1) are compactly arranged and covered with a thin cuticle. The cells of the abaxial epidermis are much smaller than those of the adaxial epidermis. Guard cells are usually found in the abaxial epidermis.

The mesophyll (or ground tissue) located between the adaxial and abaxial epidermal layers consists of palisade and spongy mesophyll. The palisade tissue is composed of columnar-like parenchyma cells which are found in the upper portion of the mesophyll. They are tightly packed and contain numerous chloroplasts (Fig.1). The spongy mesophyll is characterized by the presence of large intercellular spaces and contains parenchyma cells, which are irregular in shape. These cells also contain chloroplasts.

The vascular tissue of the leaf is found throughout the mesophyll, where it forms bundles of cells. The xylem is found in the upper portion of the bundle and is easily distinguished by the secondary wall thickenings present in these cells. The phloem is present in the lower part of the bundle, and its cells are much smaller than those of the xylem.

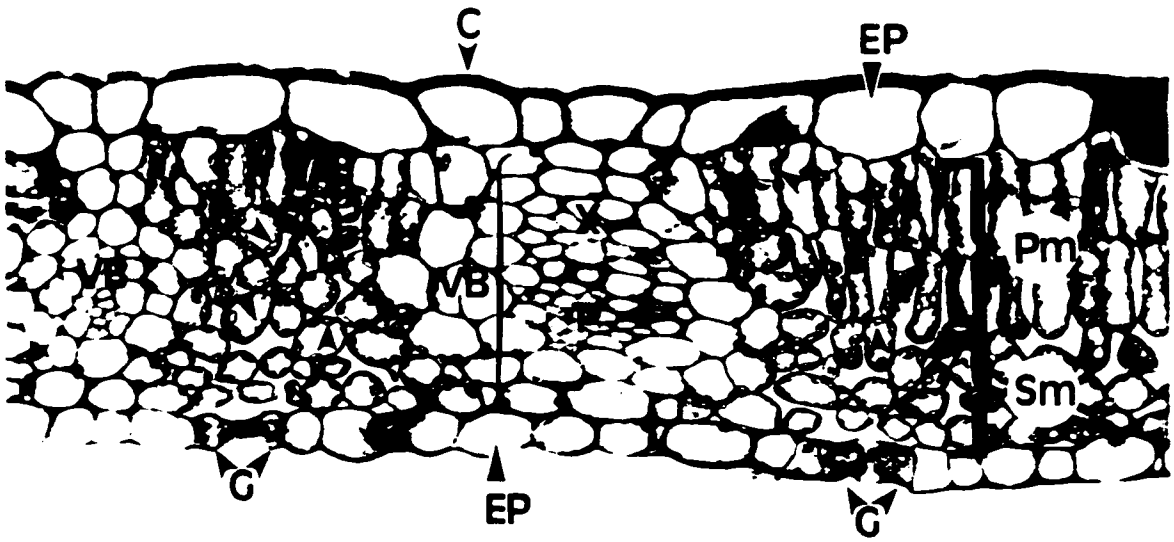
3.2 Galls of *Diplolepis rosaefolii*

3.2.1 External morphology of galls

The galls of *D. rosaefolii* are found on branches of the main vein of rose leaflets

Fig. 1. Cross section of a mature leaflet of *Rosa virginiana*. Note the cuticle on the adaxial epidermis, the anatomy of the vascular bundle (Vb), the arrangement of the chloroplast-containing (arrowheads) palisade (Pm) and spongy (Sm) mesophyll, and the presence of guard cells (G) in the abaxial epidermis. Bar = 25μm

G = guard cells, C=cuticle, EP=epidermis, P=phloem, Pm = palisade mesophyll, Sm = spongy mesophyll, VB = vascular bundle, X=xylem



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close to the midvein. Many galls are arranged in clusters or in a line adjacent to the primary vein. The majority of galls collected were found on the three most distal leaflets, but occasionally galls were found on the more proximal ones. The galls are lenticular and protrude more from the adaxial surface of the leaflet than from the abaxial surface. The gall appears smooth.

Newly initiated galls of *D. rosaefolii* (0.5 to 1 mm in diameter) are of the same color or slightly lighter than that of the leaflets and are therefore difficult to distinguish (Fig.2). They are present on the rosebushes throughout the summer months (June to September). As the galls begin to grow, the tissue under larval influence is a lighter green than in the remaining leaflet (Fig. 3), and a red spot may be visible in the center of the gall on the adaxial surface of the leaflet (Fig. 4). Most galls turn completely red as they mature (Fig. 5), while others are yellow or only partially red. The gall color is visible on both surfaces of the leaflet, although the adaxial coloration is often more vivid.

3.2.2 External morphology of larvae

Young larvae of *D. rosaefolii* are small (approximately 1.5 mm in length). The body segments appear similar in size except for the smaller most caudal segment (Fig. 6). Early instars do not have antennae but have mandibles (Fig. 7). The head segment is drawn out anteriorly, giving it a muzzle-like appearance and it has a dark colored ridge on the dorsal surface above the muzzle (Fig. 7). Spines are present on the ventral surface of the abdomen. As the larva grows, the head segment becomes small in relation to the rest of the body (Fig. 8). The final instar has peg-shaped antennae and the body is tapered

Figs. 2-5. Macroscopic surface features of different stages in the development of *Diplolepis rosaefolii* galls. Bar = 1.0 mm

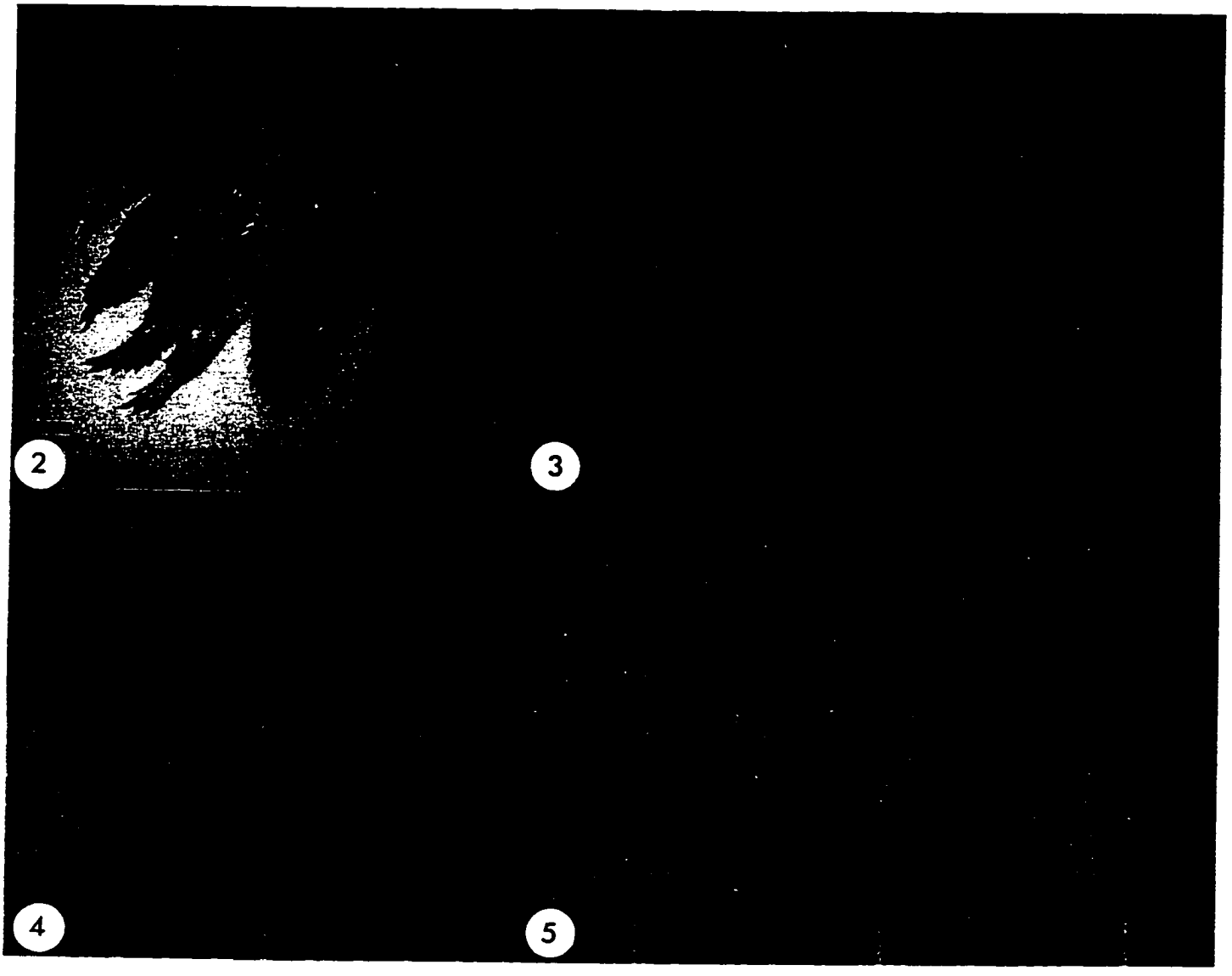
Fig. 2. Newly initiated *D. rosaefolii* galls (arrowheads) on young leaflets of *Rosa virginiana* leaves.

Fig. 3.Young galls of *D. rosaefolii* (arrowheads) on *R. virginiana* leaflets.

Fig. 4. Adaxial view of a gall (bracket) prior to maturation.

Fig. 5. Adaxial view of a mature gall of *D. rosaefolii*. Note the strong red pigmentation.

Lf = leaflet of a compound leaf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Figs. 6-9. *D. rosaefolii* larval stages. Bar = 1.0 mm

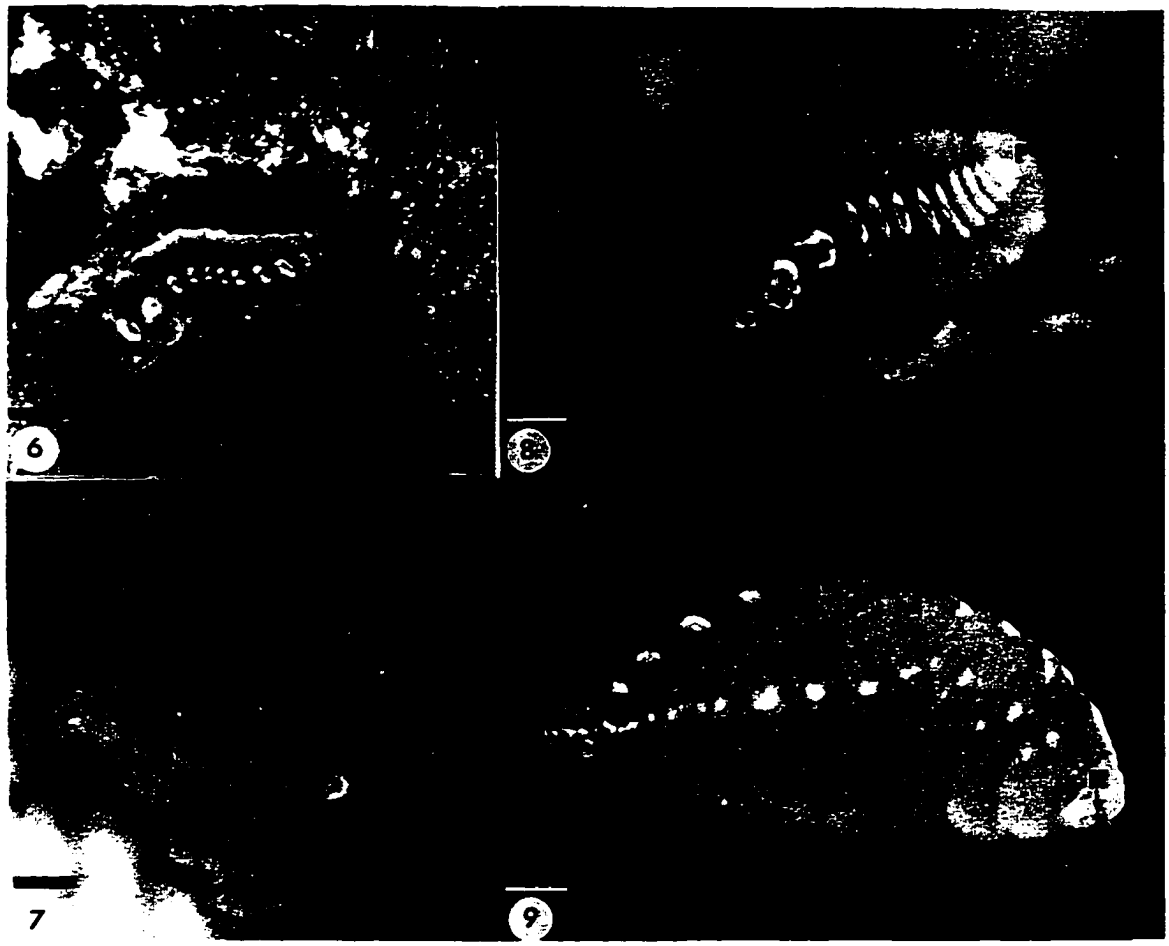
Fig. 6. Newly hatched *D. rosaefolii* larva. The 12 body segments (arrowheads) are similar in size. Note the mandibles (M) and the muzzle-like appearance of the buccal area (B).

Fig. 7.Young *D. rosaefolii* larva showing the mandibles (M) and the dark colored ridge (arrowhead) on the dorsal surface of the head.

Fig. 8. Maturing *D. rosaefolii* larva showing proportional differences between body segments, especially the small size of the anterior (A) and posterior (P) segments in relation to the rest of the body.

Fig. 9. Mature *D. rosaefolii* larva showing markedly different sized body segments. Dark colored spines (arrowheads) are present on the ventral surface and mandibles (small arrow) are visible on the head segment.

A = anterior, B = buccal area, M = mandible, P = posterior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posteriorly and anteriorly so that the head and tail segments appear smaller in diameter than their adjacent segments (Fig. 9). At this stage of development there are 12 body segments and the spines on the abdomen are darkly pigmented.

3.2.3 Developmental morphology of galls of *Diplolepis rosaefolii*

3.2.3.1 Stage 1: Initiation

Eggs are found on immature leaflets ranging in size from 5 to 10 mm. These leaflets are usually in the process of unfolding as the compound leaf expands, and the mesophyll cells are not yet differentiated into palisade and spongy mesophyll. The eggs, which range from 1.5 to 2.6 μm in length, are laid on the abaxial side of the leaflets along secondary veins (Figs.10 and 11). The ovipositional fluid surrounds the distal pole of the eggs (opposite the egg stalk) and attaches the eggs to the leaflets (Figs.12, 13, and 14). The egg is attached to a single epidermal cell, although the ovipositional fluid covers a number of cells (Fig. 15).

Changes are first observed in the cells adjacent to the ovipositional fluid at the point of attachment (Fig. 15). These cells contain fragmented vacuoles, and the nucleus and nucleoli appear enlarged in relation to those in adjoining cells (Fig. 15). As development progresses, a pad of tissue forms under the egg attachment site (Figs. 16 and 17). Subsequently, a chamber is seen under the egg and a pathway is formed through which the larva will enter the chamber after it hatches (Figs. 18 and 19).

Figs. 10-13. Arrangement of *D. rosaefolii* eggs on the abaxial surface of a leaflet.

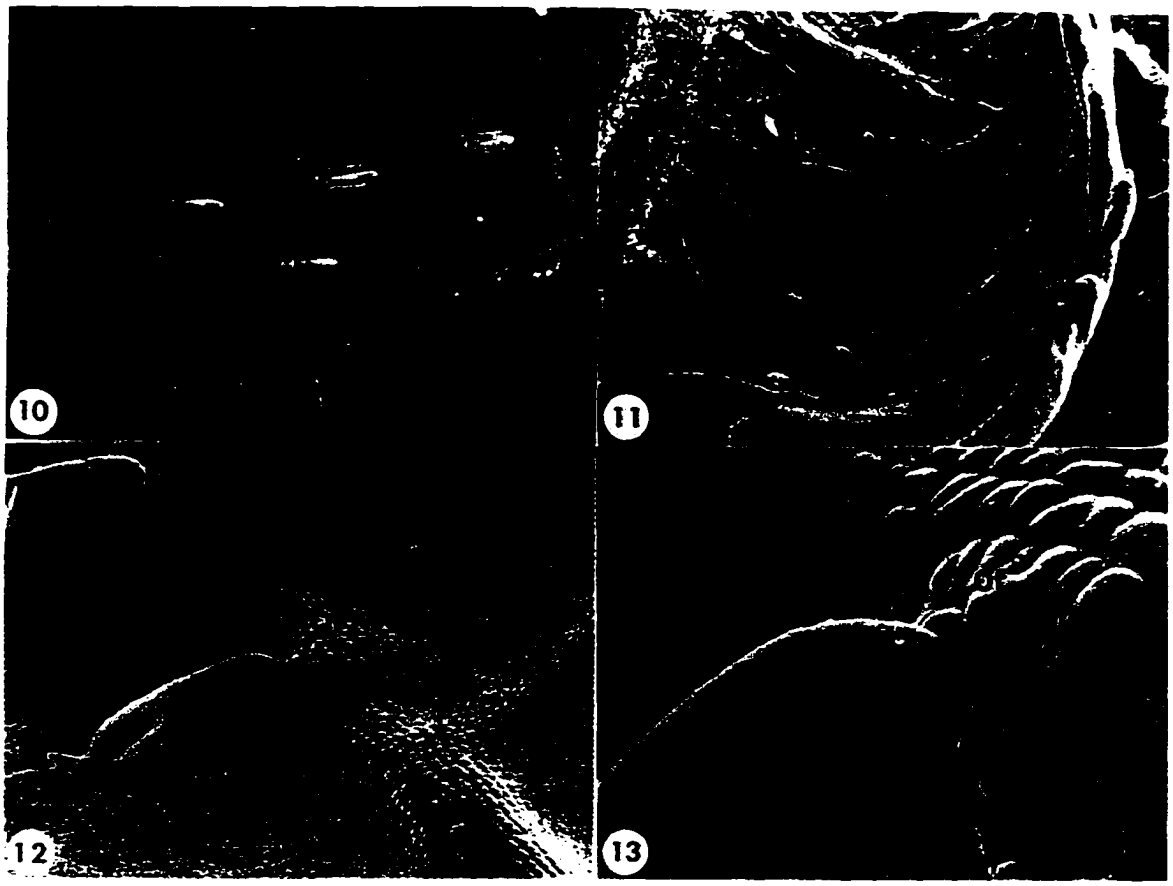
Fig. 10. Light micrograph of *D. rosaefolii* eggs (E) showing their location on the secondary veins of a leaflet. Bar = 0.3mm

Fig. 11. Scanning electron micrograph of a stage similar to that in Fig. 10 showing pedicels (arrowheads) of the eggs (E). Bar = 0.2mm

Fig. 12. Scanning electron micrograph showing the site of egg attachment (arrowhead) on the secondary vein. Bar = 50μm

Fig. 13. Higher magnification of Fig. 12 showing ovipositional fluid (Of) at the site of attachment of the egg (E) to the leaflet. Bar = 230μm

E = egg; Of = ovipositional fluid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Figs. 14-19. Stage 1: Initiation of *D. rosaefolii* galls.

Fig. 14. Cross section of a leaflet showing the location of the point of attachment of an egg (E) on the abaxial epidermal layer. Bar = 25μm

Fig. 15. Higher magnification of a stage similar to that in Fig. 14 showing cellular changes in the vicinity of the point of attachment of the egg (E) to the leaflet (area delimited by arrowheads). Note the dense cytoplasm, reduced size of the vacuole (V), and prominent nucleoli in these cells in relation to surrounding tissue. Bar = 10μm

Fig. 16. Low magnification of cross section of a folded leaflet blade showing the pad of tissue (arrowheads) which forms on either side of the attachment site of the egg (E). Bar = 0.2mm

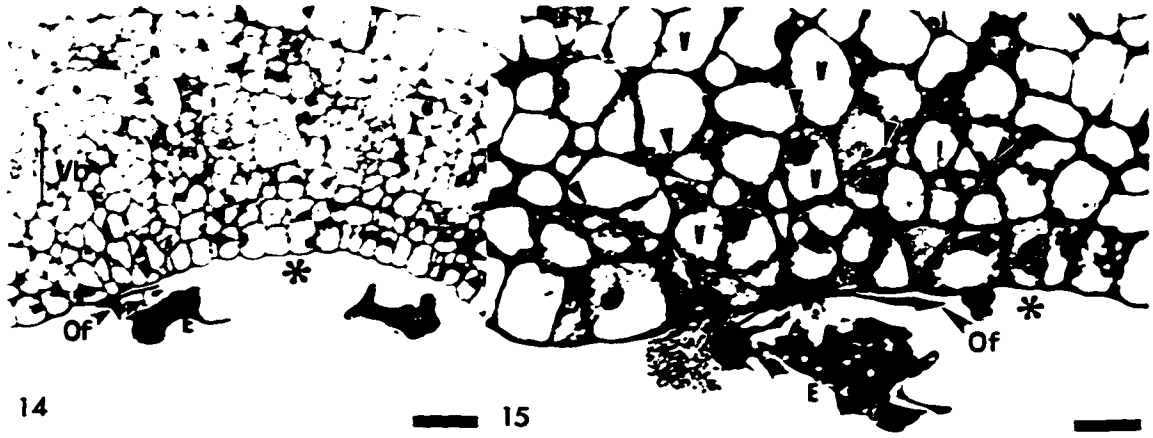
Fig. 17. Detail of the site of attachment of the egg (E) as depicted in Fig. 16 showing fragmented vacuolation (arrowheads) of the adjacent cells. Bar = 10μm

Fig. 18. Low magnification of the final phase in the initiation stage. Note the presence of a chamber (LC) adjacent to the site of larval entry (arrowhead). Bar = 50μm

Fig. 19. Detail of Fig. 18 showing the presence of lysed cells (arrowheads) surrounding the chamber (LC). Bar =10μm

E = egg, LC = larval chamber; Of = ovipositional fluid, V = vacuole, Vb = vascular bundle.

* = abaxial surface of leaflet



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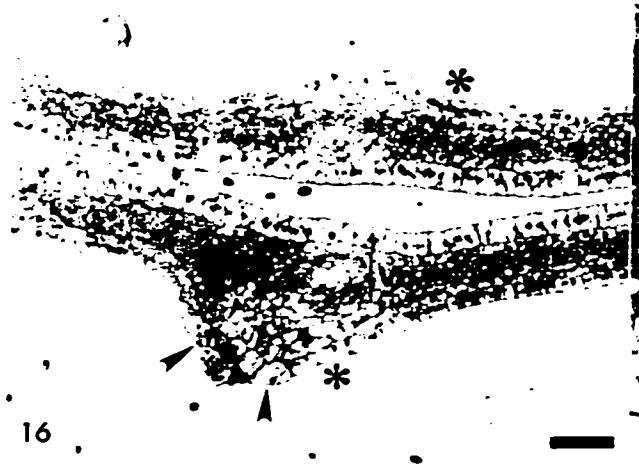

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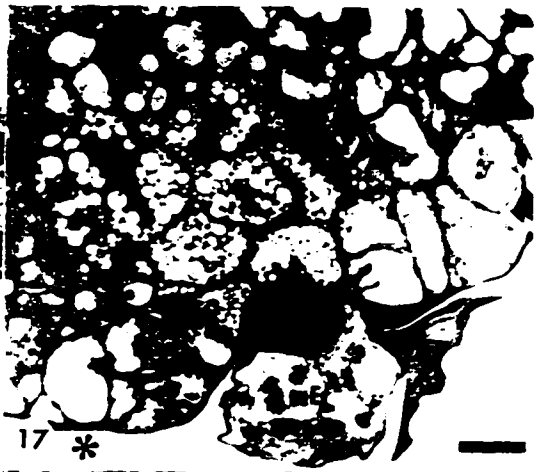

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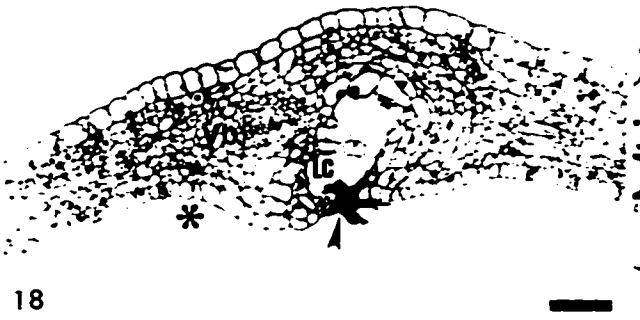

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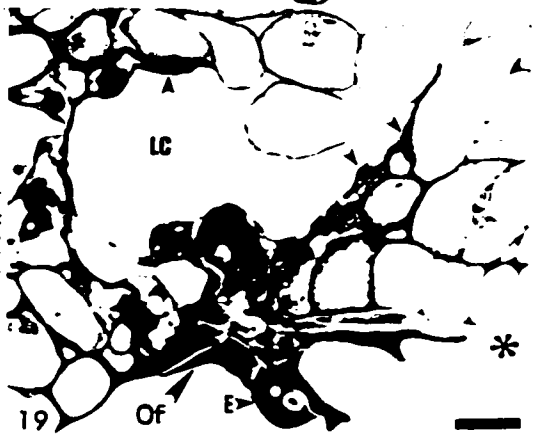

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3.2.3.2 Stage 2: Nutritive tissue formation

Once the larva enters the chamber, the larval pathway becomes obscured (Figs. 20 and 21). The egg chorion (casing) remains on the outer surface of the leaf (Fig. 20). As development continues, a group of darkly staining cells are visible on one side of the larval chamber (Figs. 20 and 22) which have characteristics similar to those of actively-dividing cells such as those found in the apical meristem (Fig. 23). Eventually, these cells come to surround the entire larval chamber (Fig. 24). The vacuole in these cells is fragmented and in cells closer to the larval chamber the vacuole is either considerably reduced or absent (Fig. 22). This change in vacuole size results in a vacuolar gradient running toward the larval chamber. The vacuoles in cells closer to the larval chamber are small and fragmented, while those in cells further away from the chamber are larger and more coalesced. A cellular gradient is also seen towards the epidermis, because the cells lining the chamber are larger than the adjacent cells (Fig. 22). The nucleus and nucleoli in these cells are much larger than those in the adjoining cells (Fig. 24). Specific staining with Sudan black B reveals large amounts of lipids sequestered in the cytoplasm of the cells closest to the larval chamber. These meristematic-like cells referred to in the literature as nutritive cells (Shorthouse 1975; Meyer and Maresquelle 1983; Bronner 1992), make up the nutritive tissue of the gall wall. The parenchyma cells, which underlie the epidermis are square in cross section (Fig. 24) and contain few chloroplasts. Starch granules are revealed in the cytoplasm of these cells by staining with Sudan black B. The vascular tissue of the leaflet is present lateral to the gall tissue (Fig. 24). As development continues, the larval chamber becomes oval in cross section (Fig. 25).

Figs. 20-25. Stage 2: Development of nutritive tissue in *D. rosaefolii* galls.

Fig. 20. Cross section of a gall showing an early stage of nutritive tissue (NT) development restricted to one side of the larval chamber (LC). Bar = 50μm

Fig. 21. Higher magnification of Fig. 20 showing the occlusion of the pathway of entry (*) of the larva. Bar = 25μm

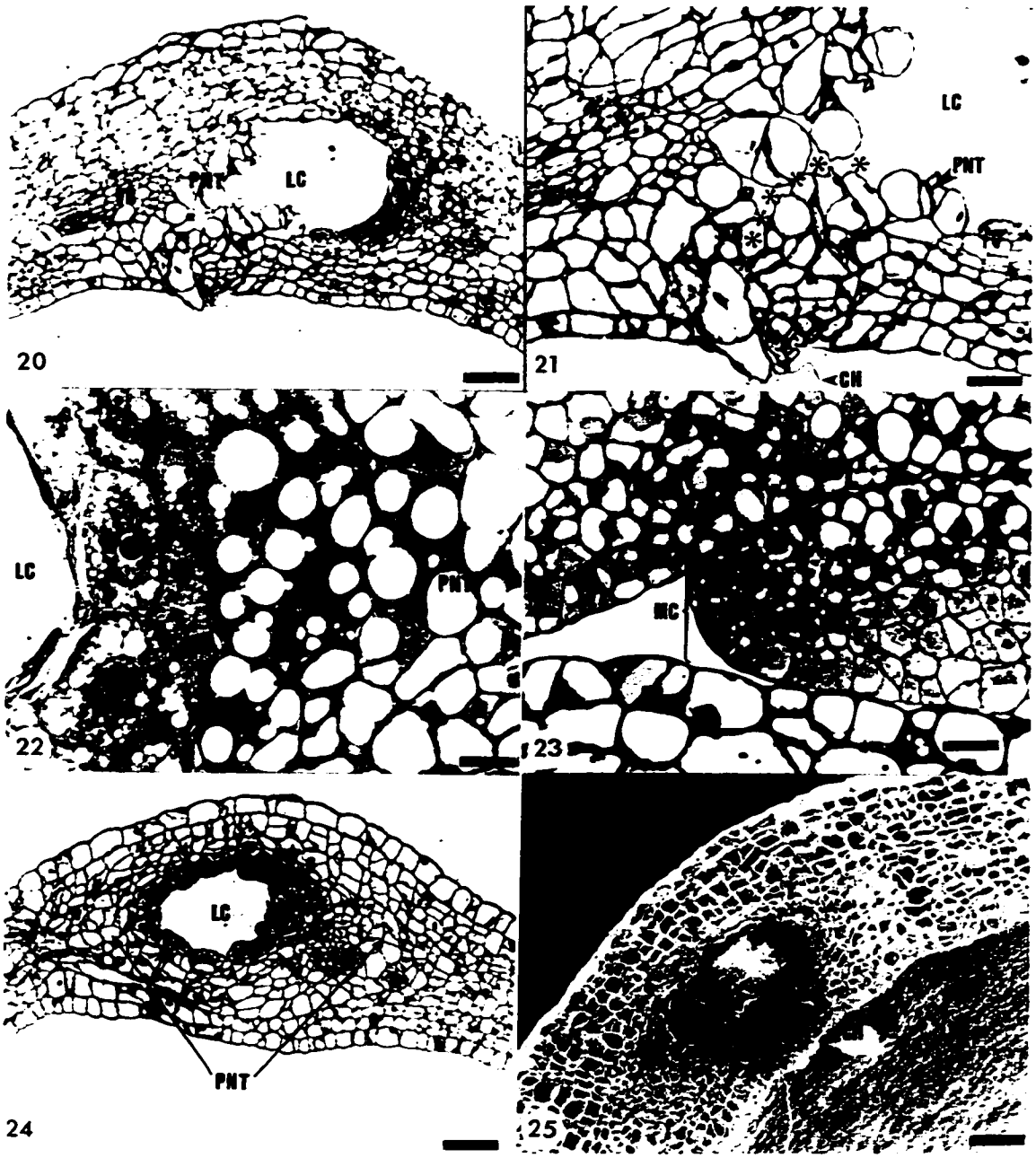
Fig. 22. Detail of the nutritive tissue showing the vacuolar gradient and variations in cell size from the larval chamber (LC) towards the periphery of the gall. Bar = 10μm

Fig. 23. Cross section of undifferentiated leaf tissue from *R. virginiana* leaf bud showing the meristematic cells (MC; for comparison with NT). Bar = 10μm

Fig. 24. Cross section of a young gall in which the nutritive tissue surrounds the larval chamber (arrowheads). Note the vascular bundle (VB) on one side of the gall. Bar = 50μm

Fig. 25. Scanning electron micrograph of a cross section through a gall. Dense cells (arrowheads) of the nutritive tissue surround the larval chamber. Bar = 50μm

CH = egg chorion, L = larva, LC = larval chamber, MC = meristematic cells, NT = nutritive tissue, PNT = parenchymal nutritive tissue, VB = vascular bundle.


PNT
LC
20
LC
PNT
21
CH
LC
PNT
22
MC
23
LC
PNT
24
25

3.2.3.3 Stage 3: Sclerification

Cells underlying the epidermis on either side of the larval chamber lay down a secondary walls forming a plate of cells above and below the chamber (Figs. 26 and 27). These layers are commonly referred to as the sclerenchymal plates (Hough 1952; Shorthouse 1975). In this study they will be referred to as the primary sclerenchymal plates because of the sequence of their development. The walls of the sclerenchyma cells thicken and the cytoplasm is restricted to a thin ring surrounding the large central vacuole (Fig. 28). Formation of the primary sclerenchymal plates is polarized, differentiating first on the adaxial side of the gall chamber and then spreading to the abaxial side (Figs. 26 and 27). Differentiation on the abaxial side of the gall chamber begins before the differentiation on the adaxial plate is completed. The sclerenchymal plates extend beyond the larval chamber in the longitudinal axis of the gall and define the area of larval influence on the leaf tissue.

At this stage of development, a layer of parenchyma cells is found between the epidermis and the primary sclerenchymal plates in addition to the layer surrounding the nutritive tissue (Figs. 26 and 27). A few cells contain chloroplasts in the outer parenchymal layer, which are found scattered throughout the tissue layer. The nutritive cells appear more abundant and the vacuolar gradient seems to be more pronounced than in the previous stage (Fig. 29). Nutritive cells appear more abundant on the sides of the gall chamber than they are above and below the chamber where only a single layer of cells may be present (Fig. 30). The sclerenchymal plates separate the gall tissues into those of the inner and outer gall; the inner gall contains nutritive and parenchymal tissue,

Figs. 26-31. Stage 3: Sclerification in *D. rosaefolii* galls.

Fig. 26. Early stage of sclerification highlighted by the presence of secondary wall thickenings (arrowheads) in cells underlying the adaxial epidermis of the leaflet. Bar = 50μm

Fig. 27. Later stage of sclerification showing primary sclerencymal plates (arrowheads) above and below the larval chamber. Bar = 50μm

Fig. 28. High magnification of sclerenchyma cells showing the secondary wall (arrowheads) and the ring of cytoplasm (large arrows) surrounding the large central vacuole (V). The separation of the cytoplasmic ring from the secondary wall is an artefact (*). Bar = 10μm

Fig. 29. Detail of nutritive tissue showing pronounced vacuolar gradient and variation in cell size from the larval chamber (LC) to the periphery of the gall. Bar =10μm

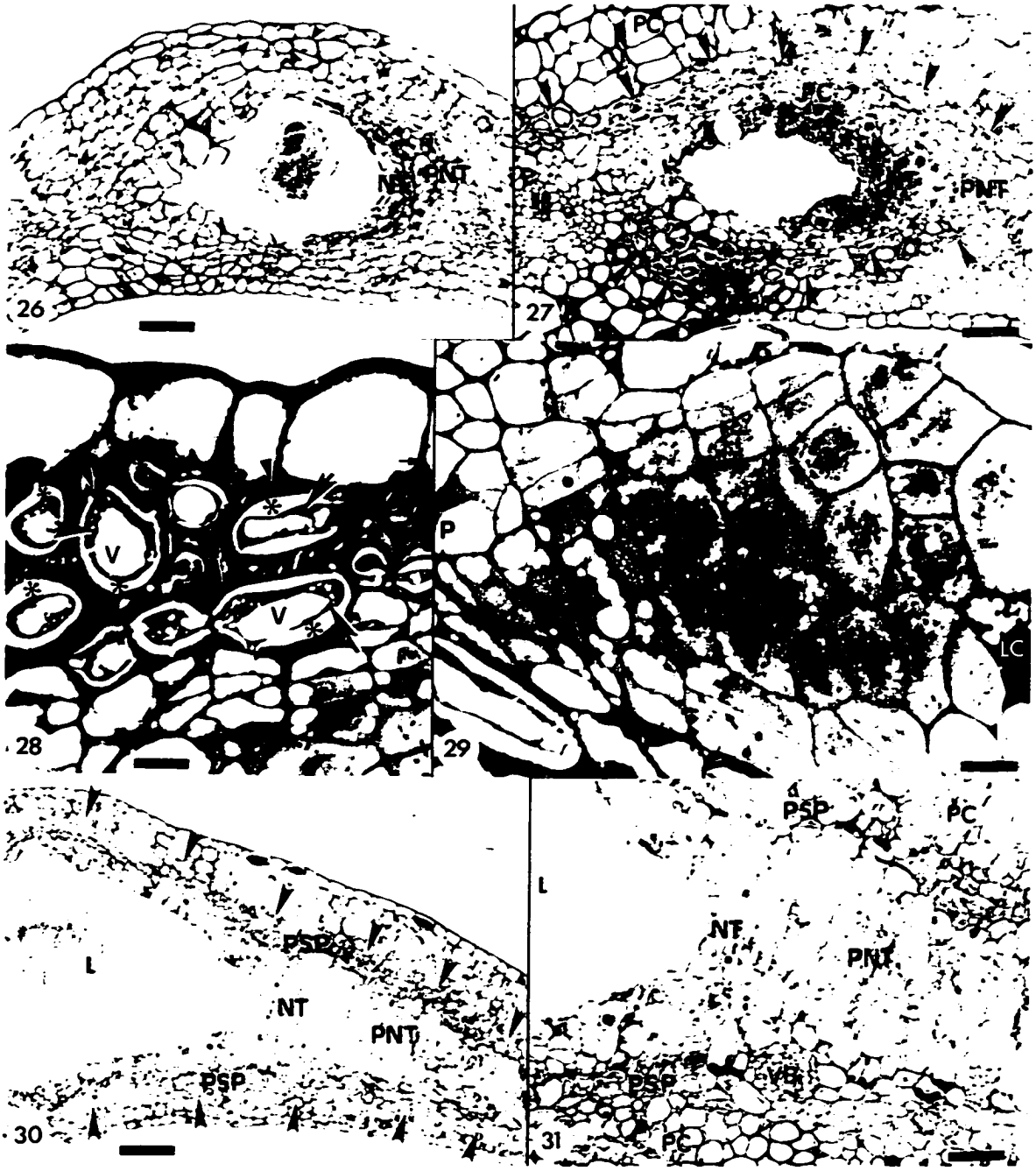
Fig. 30. Cross section through a gall chamber showing extension of the primary sclerenchymal plates (PSP; arrowheads) to the periphery of the gall. Bar = 0.1mm

Fig. 31. Higher magnification of Fig. 30 showing details of the nutritive tissue. Note the parenchyma-like nature of the cells between the primary sclerenchymal plates. Bar = 50μm

L = larva, LC = larval chamber, NT = nutritive tissue, PC = parenchyma cells,

PNT = parenchymal nutritive tissue, PSP = primary sclerenchymal plate,

VB = vascular bundle, V = vacuole.


PC
NT
PNT
26
27
V
V
28
P
LC
29
PSP
PC
L
NT
PNT
L
PSP
NT
PNT
PSP
30
PSP
PC
31

and the outer gall contains parenchymal and dermal tissue. Vascular tissue is sometimes present in the lateral regions of the lower gall wall below the primary sclerenchymal plate, but it is not seen in central regions of the wall (Fig. 31).

3.2.3.4 Stage 4: Maturation

At this stage of development of the gall, the walls of the cells in the primary sclerenchymal plate appear thicker and the size of the cell lumen is reduced when compared to cells in the previous stage (compare Figs. 32-34 to Figs. 27, 30, and 31). A new layer of sclerenchyma cells is visible adjacent to the primary plates toward the outer surface of the gall (Fig. 34). These sclerenchyma cells form the secondary sclerenchymal plates. The secondary plate in the adaxial gall wall (Fig. 33) appears to have more cell layers than the plate in the abaxial gall wall (Fig. 34). A slight undulation of this plate is visible in the adaxial wall (Figs. 32 and 33). Parenchyma cells are present between the primary and secondary plates of both walls and are also found adjacent to the epidermis in the adaxial gall wall (Figs. 33 and 34). Vascular tissue may be present in the lateral portions of the lower gall wall between the primary and secondary plates (Fig. 34).

In the early part of this stage, the nutritive tissue appears more highly vacuolated and less extensive than in previous stages (compare Fig. 35 with Figs. 22 and 29). Secondary wall thickenings appear in the parenchyma at the lateral extremes of the gall wall. Eventually, this sclerenchyma meets with the sclerenchyma of the primary plates to form a ring of sclerenchyma around the chamber. A portion of this ring is shown in Figure 36. As maturation continues, the nutritive tissue and parenchyma surrounding the

Figs. 32-37. Stage 4: Maturation of *D. rosaefolii* galls.

Fig. 32. Early stage in maturation showing different tissue types. Bar =0.25mm

Fig. 33. Higher magnification of the adaxial gall wall in Fig.32. Note the occlusion of the lumen (arrowheads) in the sclerenchyma of the primary plates (PSP) and the slight undulation of the plate. Bar = 50μm

Fig. 34. Higher magnification of the abaxial gall wall in Fig. 32. No guard cells are present in the epidermis. Note the smaller number of cell layers in the lower secondary plate (SSP) in comparison to the plate of the adaxial wall in Fig. 33. Bar = 50μm

Fig. 35. Higher magnification of the nutritive tissue (NT) found laterally in the gall from Fig. 32. At this stage of development the nutritive cells appear to be highly vacuolated. Bar = 50μm

Fig. 36. Detail of the lateral sclerenchyma (between the two rows of arrowheads) present in the gall wall at later stages of maturation. Bar = 0.1μm

Fig. 37. Mature abaxial gall wall. The larva has consumed the nutritive tissue and parenchymal tissue. Consequently the chamber (LC) is lined by cells of the primary sclerenchymal plates (PSP). Bar = 50μm

L = larva, LC = larval chamber, NT = nutritive tissue, PSP = primary sclerenchymal plate, SSP = secondary sclerenchymal plate, VB = vascular bundle..


LC
MT
L
PSP
VB
32
SSP
PSP
LC
MT
33
L
LC
MT
PSP
SSP
34
LC
MT
PMT
35
LC
MT
36
SSP
PSP
LC
37

larval chamber are consumed by the larva until the chamber is surrounded by the lateral and primary plate sclerenchyma (Figs. 36 and 37). A summary of changes in the tissue layers during the development is presented in Table 1.

3.2.4 Statistical analysis of *D. rosaefolii* gall cells

A one way analysis of variance was used to determine whether there was a difference between stages for each cell type. It revealed that the change in cell size throughout the developmental stages for parenchyma and sclerenchyma of the primary plates was significant ($p = 0.014$ and $p = 0.0001$ respectively; Fig. 38). However, the diameter of the cells in the nutritive tissue did not change significantly during development ($p = 0.109$; Fig. 38).

A multiple comparison of the cell means using Fisher's Least Significant Difference was performed to determine within each cell type where the differences occurred Minitab 1994). The changes in parenchymal cell size were significant from stage 2 to late stage 3 and to stage 4 (Table 2). However, no significant difference in parenchymal cell size was found between stage 2 and early stage 3, or between stages early stage 3, late stage 3, and stage 4 (Table 2). Significant differences were found between all stages of sclerenchymal cell development (Table 2). A significant difference was not detected between any of the stages of development in the nutritive tissue (Table 2).

Table 1. Summary of developmental changes in tissue layers of *D. rosaefolii* galls.

Diplolepis rosaefolii	Portion of gall	Stage 1 Initiation	Stage 2 Nutritive tissue formation	Stage 3 Sclerification	Stage 4 Maturation
Parenchymal tissue layer	inner gall	-cells have fragmented vacuoles -nucleus & nucleoli enlarged	-starch granules in cells -cells square in cross section -few chloroplasts	few chloroplasts	cells lost in later stage
	outer gall			no chloroplasts	cells present
Nutritive tissue layer		not present	-reduced vacuole -nucleus & nucleoli enlarged -large amount of lipids	-more cells on sides of chamber than above and below	-cells more highly vacuolated -cells lost in latter part of stage
Sclerenchymal tissue layer	primary plate	not present	not present	-thin secondary walls -thin ring of cytoplasm -large central vacuole	-thick secondary walls -lumen reduced
	secondary plate	not present	not present	not present	-thin secondary walls
	lateral	not present	not present	not present	-forms at sides of chamber -meets primary plate

Figure 38. Mean cell diameters for tissue types in *Diplolepis rosaefolii* galls at various stages of development.

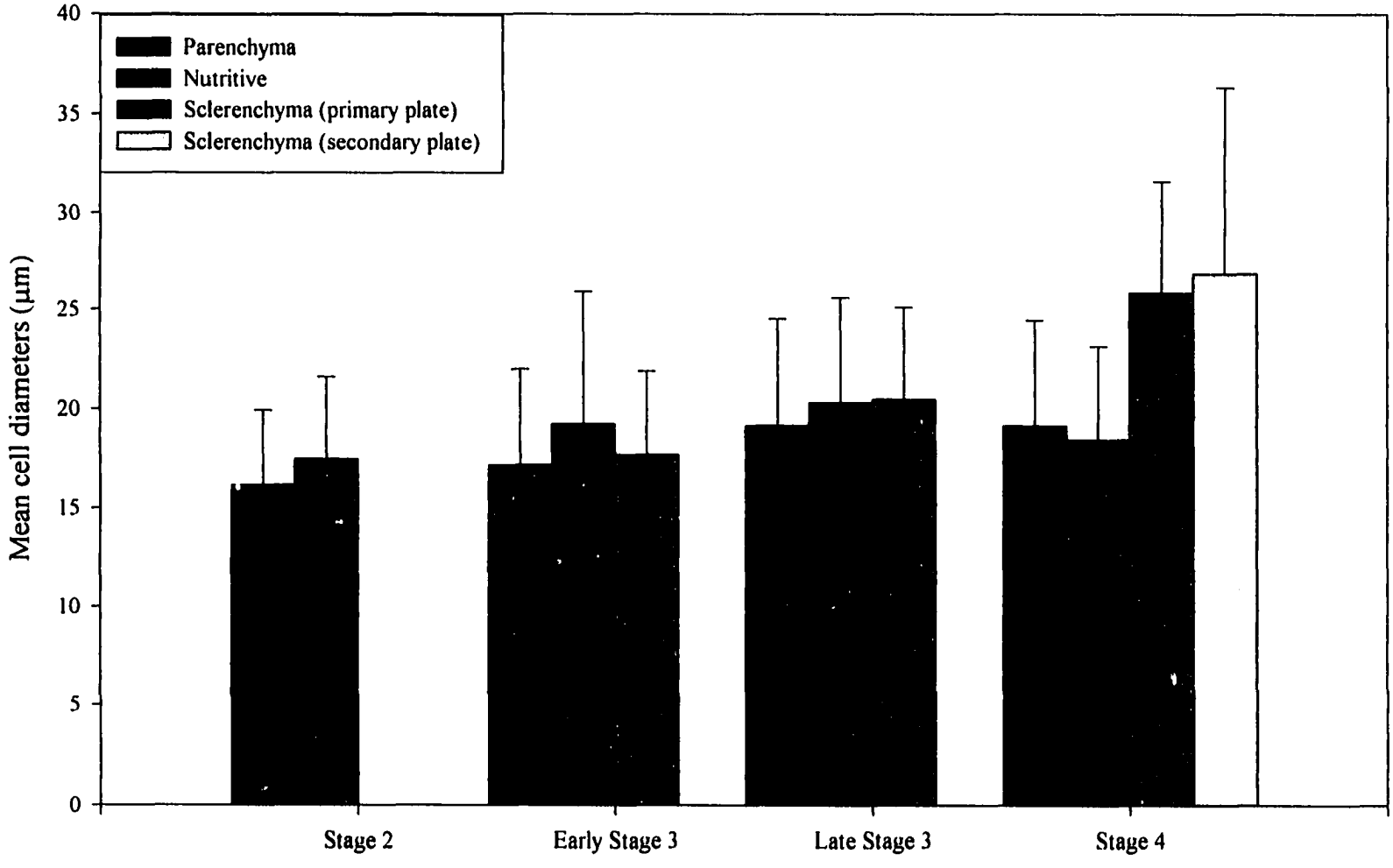


Table 2. Analysis of mean cell diameters in various tissue layers of *D. rosaefolii* galls.

Stage of development	Mean cell diameters (μm)[a]			
	Cell type			
	Parenchyma	Nutritive	Sclerenchyma (primary plate)	Sclerenchyma (secondary plate)
stage 2	16.17a	17.50a	NA	NA
Early Stage 3	17.19ab	19.24a	17.70a	NA
Late Stage 3	19.12b	20.31a	20.48b	NA
Stage 4	19.13b	18.44a	25.84c	26.84
p value for LSD (F)	0.0136 (3.67)	0.110 (2.05)	0.0001 (28.49)	NA

[a]Mean in columns followed by the same letter are not significantly different; $p \leq 0.05$

3.3 *Periclistus*

3.3.1 External morphology of galls

Galls containing *Periclistus* eggs appear more flattened than when the inducer is present in the gall. This is similar to the galls in which the *D. rosaefolii* larva is host to a parasite and is therefore no longer actively feeding on the gall tissue (LeBlanc 1995). Galls containing actively feeding *Periclistus* larvae protrude from the adaxial surface of the leaf much more than those containing *D. rosaefolii* larvae (Fig. 39). The adaxial surface of the gall is irregular and the central portion is raised, forming a semicircular projection from the leaf. In young galls, the central upraised portion of the gall is characterized by a lightly pigmented area (yellow or green) surrounded by a ring of darkly pigmented red or green tissue (Fig. 40). Around the darkly pigmented area there is a flat ring of yellow tissue. At maturity the entire gall turns red (Fig. 41).

3.3.2 External morphology of the larvae

Young *Periclistus* larvae have a well-defined head with mandibles and a pair of spine-like projections on either side of the dorsal surface of the head. The body segments appear to decrease in diameter from the head to the caudal end (Fig. 42). As the larvae mature, the segments become more uniform in size (Fig. 43) and the dorsal surface becomes divided into a series of three ridges stretching across each segment (Fig. 44). The centrally located ridge appears larger than the peripheral ridges.

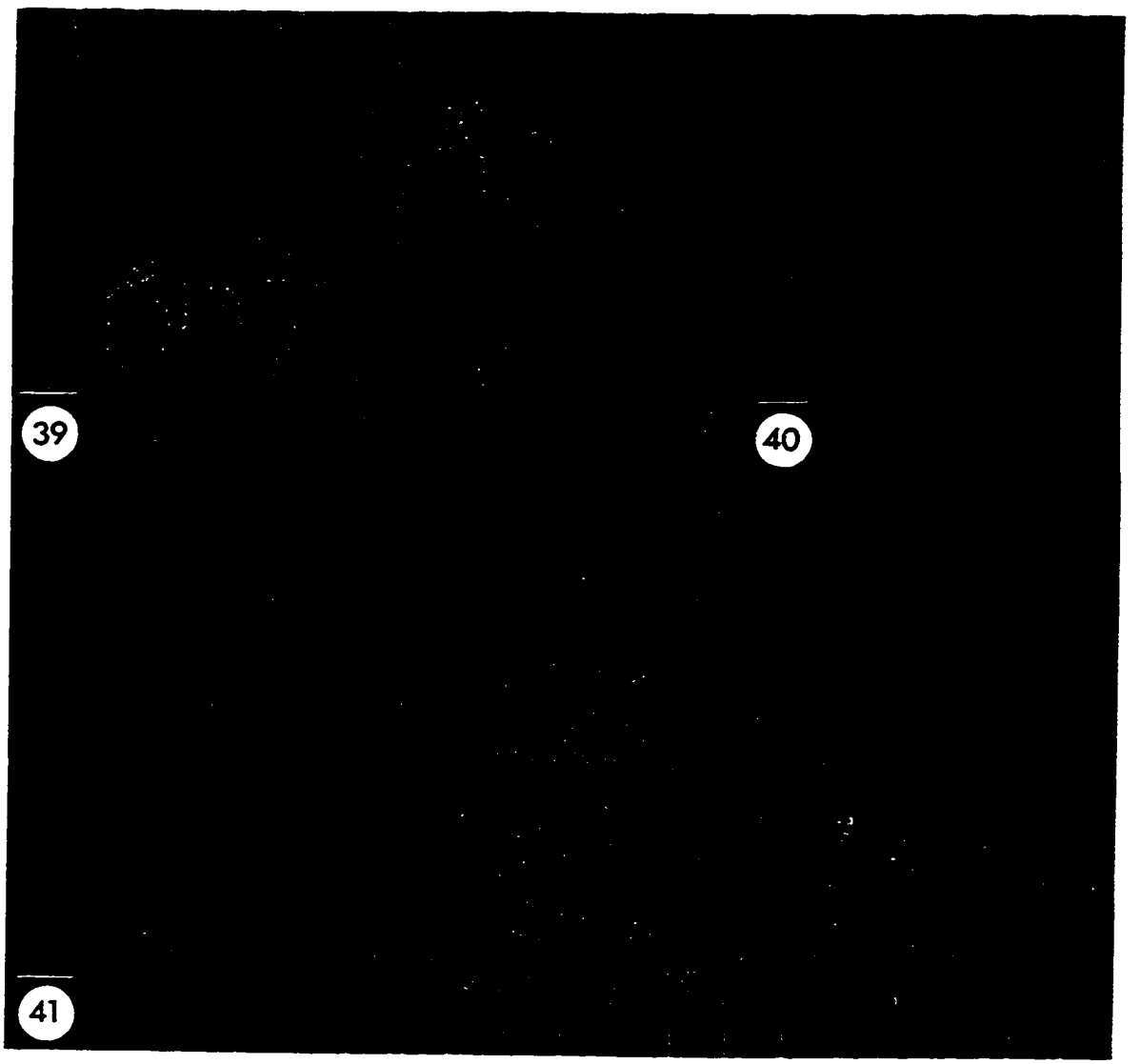
Figs 39-41. Macroscopic surface features of stages in the development of *Periclistus*-inhabited galls. Bar = 0.1 mm

Fig. 39. Galls containing *D. rosaefolii* (D) and *Periclistus* (P) larvae. The *Periclistus*-containing gall protrudes from the surface of the leaf much more than the *D. rosaefolii*-containing gall.

Fig. 40. Young *Periclistus*-inhabited gall, showing lightly pigmented central area on the exposed portion of the gall.

Fig. 41. Darkly pigmented, mature galls inhabited by *Periclistus* are illustrated.

D = *D. rosaefolii* gall, P = *Periclistus* gall


39
40
41

Figs. 42-44. *Periclistus* larval stages.

Fig. 42. Newly hatched *Periclistus* larva. The body segments (arrowheads) are progressively smaller from the anterior to the posterior segments. Bar = 0.15 mm

Fig. 43. Maturing *Periclistus* larva showing mandibles (M) and well-defined body segments. Bar = 0.33 mm

Fig. 44. Mature larvae of *Periclistus* showing the mandibles (M) and a series of three ridges (1, 2, 3) stretching across the dorsal surface of each segment. Bar = 0.33 mm

M = mandibles

42
43
44

3.3.3 Gall development

Eggs of *Periclistus* can be deposited in galls induced by *D. rosaefolii* during all but the earliest stages of development. This wide developmental window, in *D. rosaefolii* gall development, during which *Periclistus* can lay eggs in the galls results in a high degree of variablility in the changes which are subsequently induced by the *Periclistus* larvae in the galls. When *Periclistus* takes over the influence of the gall at an early stage (during stage 2 or 3), more changes are evident than when the gall is inhabited at stage 4. The initial changes induced by *Periclistus* larvae are the same regardless of the stage in *D. rosaefolii* gall development at which the *Periclistus* eggs are laid in the gall.

3.3.3.1 Egg deposition and initial changes in the galls

One or more eggs of *Periclistus* are laid in the *D. rosaefolii* gall chamber (Figs. 45, 46, and 47). The egg stalk is attached to a single cell of the innermost gall wall by ovipositional fluid (Figs. 47 and 48). Nutritive tissue is not present around the gall chamber at this stage (Figs. 46 and 47). A large amount of parenchymal tissue is present on either side of the larval chamber (Fig. 46). These parenchyma cells are rectangular in cross section and are devoid of chloroplasts. Parenchyma cells are also found adjacent to the epidermis (Fig. 46) and contain chloroplasts.

3.3.3.2 Further changes in gall tissues induced by *Periclistus* larvae

3.3.3.2.1 Nutritive tissue

Once the *Periclistus* larvae hatch and begin to feed, nutritive tissue is seen

Figs. 45-48. *Periclistus* egg attachment and initial changes in *D. rosaefolii* galls.

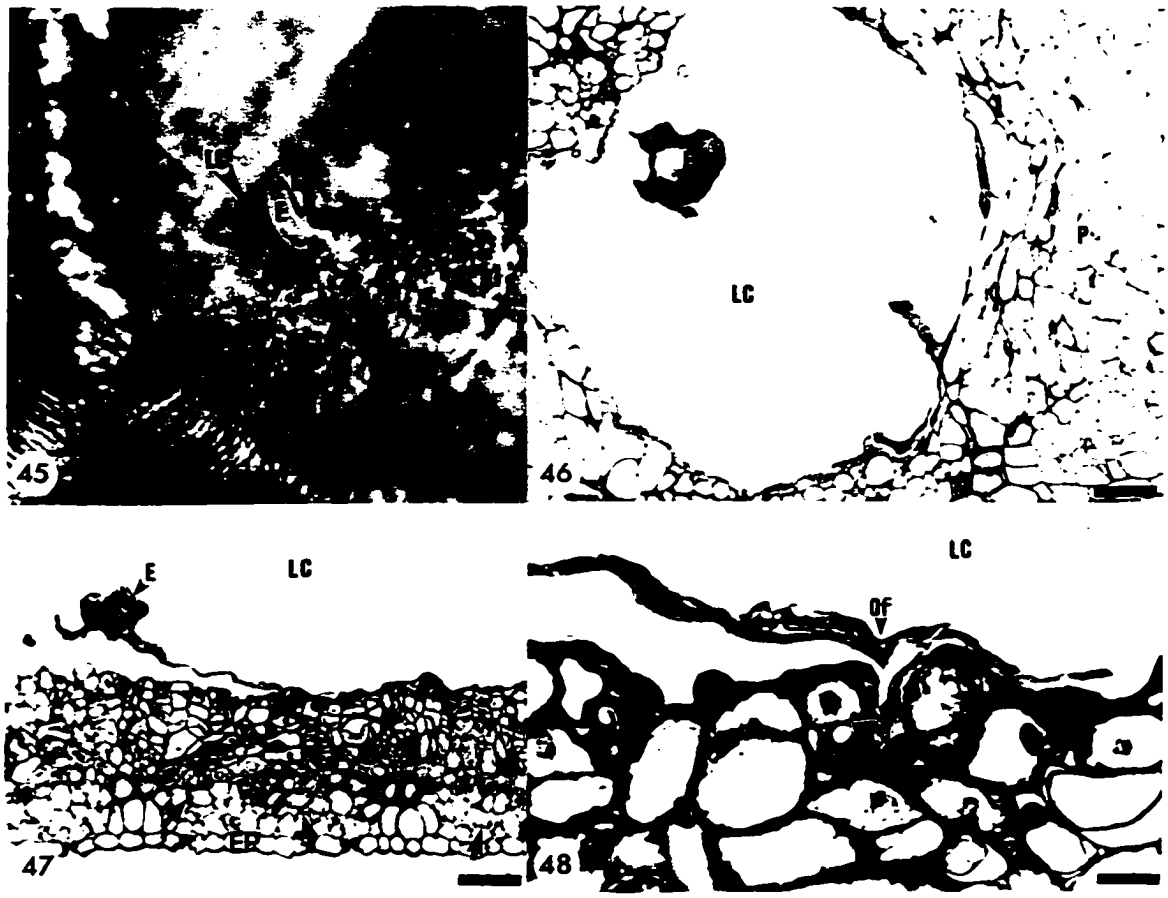
Fig. 45. Dorsal view of *D. rosaefolii* gall chamber (LC) showing *Periclistus* eggs (E). Bar = 0.17 mm

Fig. 46. Cross section of *D. rosaefolii* gall, showing enclosed *Periclistus* egg. Note the absence of nutritive tissue surrounding the chamber and the presence of thin-walled parenchyma (P) peripheral to the chamber (LC). Bar = 10 μm

Fig. 47. Cross section of the abaxial gall wall showing attachment of *Periclistus* egg (E). Note the chloroplasts (arrowheads) in the parenchyma cells. Bar = 50μm

Fig. 48. Higher magnification of Fig. 47 to show details of attachment site of a *Periclistus* egg inside the gall chamber (LC). The egg (not shown) is attached to a single cell (arrowhead) by the ovipositional fluid (Of). Bar = 10 μm

E = egg, EP = epidermis, LC = larval chamber, P = parenchyma, Of = ovipositional fluid


LC
E
45
LC
P
46
E
LC
47
LC
OF
48

surrounding the larval chamber (Fig. 49). Hatching of multiple *Periclistus* eggs in one gall can result in galls containing unhatched eggs and larva at the same time. The nutritive tissue consists of multiple files of cells which appear evenly dispersed around the larval chamber (Fig. 49). The cells are rectangular in cross section (Fig. 50). A decreasing vacuolar gradient appears to be present in the nutritive cells, with the largest vacuoles in the cells abutting the parenchyma and the smallest vacuoles in the cells next to the larval chamber (Fig. 50).

In later stages of development, the nutritive cells appear more plentiful at the top and bottom of the larval chamber, and only a few layers of cells are present on either side of the larval chamber (Fig. 51). Eventually, in even later stages of development nutritive tissue is absent around the chamber (Fig. 52).

3.3.3.2.2 Parenchymal tissue

Parenchymal tissue occurs around the nutritive tissue in the inner gall wall and under the epidermis in the outer gall wall (Fig. 49). The amount of parenchyma in the inner gall wall appears more extensive on either side of the gall chamber than at the top and bottom of the chamber (Fig. 51). The parenchyma cells at the top of the chamber appear more numerous than those at the bottom (Figs. 51 and 52). The parenchyma cells of the inner gall wall appear rectangular in cross section and have a thin layer of cytoplasm surrounding a large central vacuole (Fig. 53). Chloroplasts are absent in these cells. The parenchyma cells in the outer gall are localized in bounded regions surrounded by the sclerenchyma cells and the epidermis. The cells are square in cross section and contain numerous chloroplasts. The chloroplasts are found in the ring of cytoplasm which

Figs. 49-54. Cross sections of *Periclistus*-inhabited galls, highlighting the nutritive and parenchyma tissues.

Fig. 49. Cross section of young *Periclistus*-inhabited gall. Although this gall has two chambers, only one is in median section (1), while the other (2) is sectioned through its parenchymal layer. Nutritive tissue (NT) surrounds the larval chamber. Bar = 0.25mm

Fig. 50. Detail of nutritive tissue of gall shown in Fig. 49. Note an increasing amount of vacuolation in cells of the nutritive tissue from the gall chamber (LC) towards the periphery of the gall (top of photo). Bar = 10μm

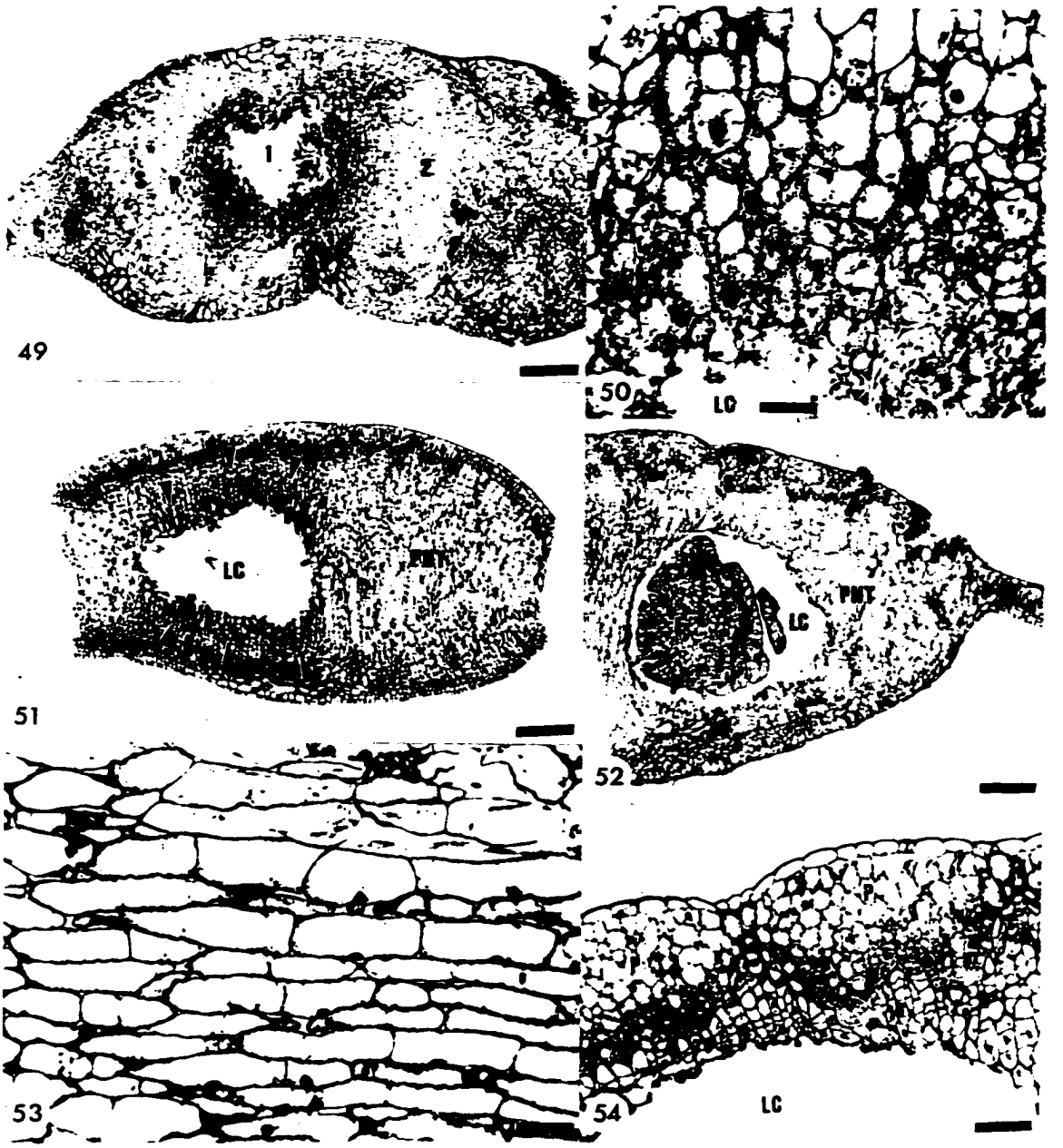
Fig. 51. Cross section of a maturing *Periclistus*-inhabited gall at a later stage of inquiline influence. At this stage, the nutritive tissue (arrowheads) is only present at the top and bottom of the gall chamber (LC). Bar = 38μm

Fig. 52. Cross section of a mature *Periclistus*-inhabited gall. The *Periclistus* larva (L) is large in relation to the size of the gall chamber (LC). Note the lack of nutritive tissue lining the gall chamber. Bar = 0.19 mm

Fig 53. Detail of thin-walled and highly vacuolated parenchyma cells lateral to the gall chamber in *Periclistus*-inhabited gall. Bar = 10μm

Fig. 54. Cross section of the adaxial wall of a *Periclistus*-inhabited gall showing the patches of parenchyma cells (P) underlying the epidermal layer. Note the presence of chloroplasts (darkly stained inclusions) in these cells. Bar = 50μm

L = larva, LC = larval chamber, NT = nutritive tissue, PNT = parenchymal nutritive tissue, S = sclerenchyma


1
2
49
50
LC
LC
51
52
LC
PHT
53
54
LC

surrounds the central vacuole (Fig. 54).

Later in development, parenchyma cells are present in the upper gall wall and on either side of the chamber (Fig. 52). However, parenchyma cells are absent in the lower gall wall except for a thin layer underlying the epidermis (Fig. 52).

3.3.3.2.3 Sclerenchymal tissue

The sclerenchymal cells in the upper gall wall form an undulating layer between the parenchymal and nutritive tissues (Fig. 55). The undulations are much more pronounced than those seen in some mature *D. rosaefolii*-inhabited galls at the stage of maturation. At various intervals along the gall wall, the sclerenchyma cells form extensions to the epidermis, thus dividing the parenchyma of the outer gall wall into a series of pockets underlying the epidermis (Fig. 56). In galls where two layers of sclerenchyma are present (Fig. 57), the outer sclerenchymal layer forms an undulating band of tissue (Fig. 58).

The sclerenchyma cells of the abaxial gall wall form a layer parallel to the gall chamber (Fig. 59). Vascular tissue is present along the length of the entire gall wall and is situated between the sclerenchyma and the nutritive tissues (Fig. 60). Sclerenchyma extends on either side and at the top of the vascular bundles thus enclosing each bundle, but these projections extend toward the nutritive tissue and not toward the epidermis as in the upper gall wall (Fig. 59).

In galls where two layers of sclerenchyma are present, the vascular bundles are found between the layers (Fig. 60). Secondary wall thickenings can be seen in the cells on

Figs. 55-60. Cross sections of *Periclistus*-inhabited galls, showing arrangement of sclerenchymal layers.

Fig. 55. Cross section of *Periclistus*-inhabited gall chamber with single sclerenchymal plate (arrowheads) above and below the larval chamber. Bar = 19μm

Fig. 56. Detail of an adaxial wall in *Periclistus*-inhabited gall, showing undulations in the upper sclerenchymal plate isolating areas of chloroplast-containing parenchyma tissue (P). Bar = 19μm

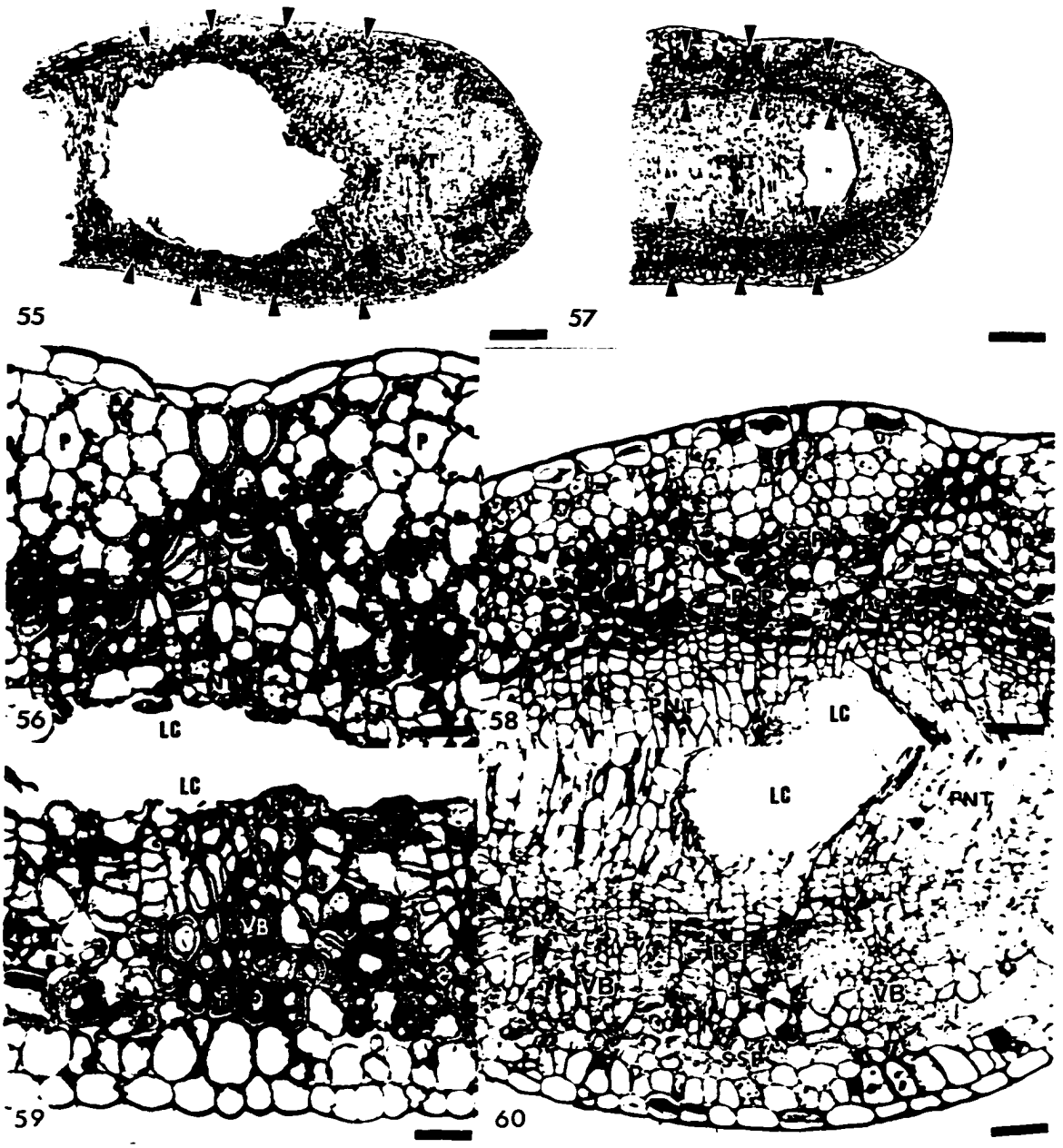
Fig. 57. Cross section of a *Periclistus*-inhabited gall with double sclerenchymal plates (arrowheads) above and below the larval chamber. Bar = 19μm

Fig. 58. Cross section of an adaxial wall in *Periclistus*-inhabited gall showing undulations of the secondary sclerenchymal layer (SSP) and the heavy deposition of secondary cell wall in the cells of the primary sclerenchymal layer (PSP). Bar = 19μm

Fig. 59. Cross section of an abaxial wall in *Periclistus*-inhabited gall with single sclerenchyma layer surrounding a vascular bundle (VB). Bar = 50μm

Fig. 60. Detail of an abaxial wall in *Periclistus*-inhabited gall with a double sclerenchyma layer. Vascular bundles (VB) are located between the two sclerenchymal plates (PSP & SSP). Bar = 50μm

LC = larval chamber, NT = nutritive tissue, PNT = parenchymal nutritive tissue, PSP = primary sclerenchymal plate, SSP = secondary sclerenchymal plate, VB = vascular bundle


55
57
56
LC
58
PNT
LC
LC
PNT
59
LC
VB
60
VB
VB

either side of the gall chamber along the boundary of the large rectangular gall parenchyma (Fig. 61). In later development, sclerenchyma is present on either side of the gall chamber (Fig. 62) and this extends to the sclerenchymal plates to surround the entire gall chamber. When both primary and secondary sclerenchymal plates are present, the lateral sclerenchyma extends to the inner plate. In galls where two or more chambers are present, the lateral sclerenchyma is only present on one side of the chamber (Fig. 49).

3.3.3.2.4 Summary of changes in *Periclistus*-inhabited galls

The *Periclistus*-inhabited gall chamber is somewhat circular, but the shape is generally irregular with many protrusions (Figs. 49, 51, 52, and 55). Tissue development in these galls is largely dependant on the stage of development of the inducer gall at the time the eggs are layed and on the number of eggs layed in the gall. Single chambered galls, containing only one larva have concentric layers of tissue surrounding the larval chamber (Fig. 51). In multichambered galls, with more than one larva present, the nutritive tissue and the inner layer of parenchyma surround each chamber. However, the sclerenchyma does not differentiate in regions between chambers (Fig. 49). Galls in which *Periclistus* eggs are laid at stage 2 or stage 3 of *D. rosaefolii* development do not contain secondary sclerenchymal plates. However, if eggs are laid at stage 4 of development, the secondary plate is retained and the undulations in the upper wall are much more pronounced than when the *D. rosaefolii* larva is present (compare Figs. 32 and 33 with Figs. 55 and 56). In all galls inhabited by *Periclistus*, vascular tissue is present in the gall wall at a very early stage of development and forms a row of bundles

Figs. 61-63. Cross sections of *Periclistus*-inhabited galls showing differentiation of lateral sclerenchyma. Bar = 38μm

Fig. 61. Initial stage of lateral sclerenchyma differentiation in *Periclistus*-inhabited gall showing secondary wall thickenings (arrowheads) in cells adjacent to the larval chamber (LC).

Fig. 62. Mature lateral sclerenchyma (LS) in *Periclistus*-inhabited gall. Note the lateral sclerenchyma meets (arrowheads) the primary sclerenchymal plate.

Fig. 63. Higher magnification of lateral sclerenchyma (LS) shown in Fig. 62. A large number of pits (small circular holes) are present in the secondary walls of the lateral sclerenchyma. The secondary sclerenchymal layer (SSP) runs adjacent to the lateral sclerenchyma and separates it from the parenchymal tissue (P).

G = guard cell, L = larva, LC = larval chamber, LS = lateral sclerenchyma, P = parenchyma, PNT = parenchymal nutritive tissue, PSP = primary sclerenchymal plate, SSP = secondary sclerenchymal plate

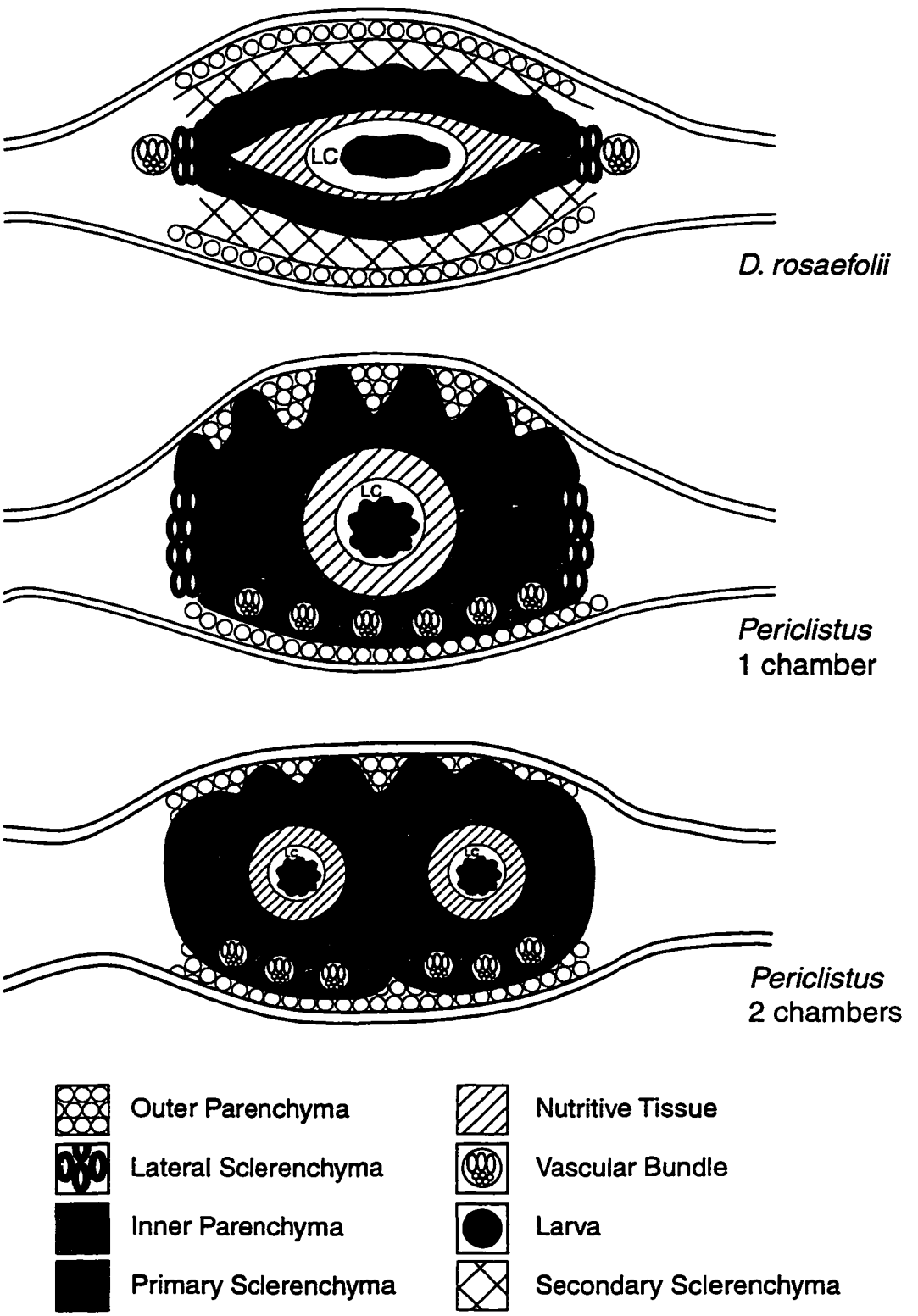

PNT
61
PNT
LC
62
LS
P
63

along the lower gall wall (Fig. 60). The vascular bundles are surrounded by sclerenchyma cells whether one or two plates are present (Figs. 59 and 60). Chloroplasts are found in the cells of the outer parenchymal layer and air spaces are present between these cells (Fig. 56). The changes induced by *Periclistus* larvae are summarized in Table 3 and illustrated in Figure 64.

Table 3. Summary of major developmental changes in galls inhabited by *D. rosaefolii* and *Periclistus* larvae.

	tissue	early development	middle development	late development
Diplolepis rosaefolii	nutritive	- cells differentiate at one side of the chamber, then the other -cells square in cross section -large amount of lipids present -cellular size gradient decreasing from chamber	-cells more abundant at sides of chamber	-cells more abundant at sides -layer lost in later stages
	parenchyma	-cells square in cross section -starch present	-few chloroplasts in cells scattered through inner layer	-inner layer lost in later stages
	sclerenchyma	-not present	-primary plate formed	-secondary plates formed -lateral sclerenchyma present: meets primary (inner) plates
	vascular	-bundles at sides of chamber	-bundles at sides of chamber	-may be bundles between the sclerenchymal plates in the lower wall at the periphery of the gall
Periclistus	nutritive	-differentiates all around chamber -rectangular in cross section -starch & lipids present -cells all same size	-cells surround larval chamber	-cells found at top and bottom of chamber only -layer lost on later stages
	parenchyma	-rectangular in cross section	-many chloroplasts in outer layer, none present in inner layer	-inner layer lost
	sclerenchyma	-may be none, 1, or 2 layers present -1 layer present: undulations in upper wall -2 layers present: undulations in outer layer of upper wall	-1 or 2 layers present	-lateral sclerenchyma present: meets inner plates
	vascular	-row of bundles in lower gall wall	-row of bundles in lower gall wall	-row of bundles in lower gall wall

Fig. 64. Graphical representaion of *D. rosaefolii* and *Periclistus*-modified galls.
L = larva, LC = larval chamber



4. Discussion

The wild rosebushes in Prince Edward Island are host to four species of *Diplolepis* gall inducers (Dr. J. D. Shorthouse, Laurentian University, personal communication). These include *D. bicolor*, *D. rosaefolii*, *D. nodulosa*, and an as yet unnamed *Diplolepis* species. All species on Prince Edward Island, except *D. nodulosa* induce galls on the leaves of wild roses. The galls of *D. rosaefolii* are more abundant and more visible than the other *Diplolepis* galls and therefore were better suited for a study of developmental morphology because galls at many different stages of development could be obtained.

4.1 Changes in leaf morphology

Any study of gall morphology must take into consideration the morphology of the organ on the specific plant where the gall occurs. In this way the changes induced by the larvae are readily discernible from the normal morphology of the organ.

When eggs of *D. rosaefolii* are laid on the leaves of *R. virginiana*, the mesophyll cells are still undifferentiated. Soon after the larva enters the gall chamber the mesophyll cells peripheral to the larval chamber are differentiated into the palisade and spongy tissues characteristic of the fully developing leaf. However, subsequent changes in tissue patterns induced by the *D. rosaefolii* larva diverge significantly from normal leaf development. Shorthouse (1975) saw similar divergence in the six *Diplolepis* species he studied. Initially, lysis in the cells in contact with the egg results in the formation of a chamber in the leaf which the larva enters upon hatching. This is followed by the

dedifferentiation of the mesophyll cells immediately surrounding the chamber, and results in the formation of meristematic-like cells of the nutritive layer. Shorthouse (1975) noted similar dedifferentiation in development of galls induced by *D. lens*, *D. nebulosa*, *D. ignota*, and *D. gracilis*. The parenchyma surrounding the nutritive cells remains undifferentiated and air spaces do not develop between the cells as would typically happen during the differentiation of palisade and spongy mesophyll. The differentiation of the sclerenchymal plates is the most notable change that takes place in the gall tissues, as sclerenchyma is not found in the normal leaf tissue except in and around the vascular bundles. The development of the gall results in the formation of tissues whose type and orientation in no way resemble that of the normal leaf. Furthermore, the gall cells appear to be larger and more abundant than the typical leaf cells resulting in a growth which is readily discernible on the surface of the leaf.

Changes in cell contents are also observed in gall tissues when compared to those of the normal leaf. Many of the epidermal cells contain a darkly staining substance in their vacuole and the parenchyma cells have very few chloroplasts. Hough (1952) and Mani (1964) both found decreased amounts of chlorophyll in gall tissue and Hough (1952) noted the presence of large amounts of anthocyanins in the vacuoles of the cells. Therefore, the red coloration of *D. rosaefolii* galls may result from decreased chlorophyll levels due to the lack of chloroplasts in the cells and the presence of anthocyanins in the vacuoles of the epidermal cells. The nutritive cells in the gall exhibit the greatest change in cell contents. These cells have an enlarged nucleus and nucleolus, as well as large quantities of lipids and many small, fragmented vacuoles. These cytological features are

similar to those found in other cynipid galls studied and are characteristic of cells which are physiologically and metabolically active (Bronner 1977).

4.2 *Diplolepis rosaefolii* gall development

The development of *D. rosaefolii* galls was similar to that described by Rohfritsch (1992) in her general model of cynipid gall development, where three stages are highlighted including i) initiation, ii) growth, and iii) maturation. This model is based on circular galls in which tissues are arranged in concentric rings around a centrally located gall chamber. Although the orientation of the tissues in lenticular galls, such as those induced by *D. rosaefolii*, is not circumscribing as is depicted in the model, the sequence of the differentiation of the various tissue layers follows the cynipid model.

Eggs of *D. rosaefolii* are attached by ovipositional fluid to a single epidermal cell on the abaxial surface of the leaf. This is consistent with other studies on *Diplolepis* species (*e.g.* Bronner 1985 on *D. rosae*; Shorthouse 1993 on *D. polita*) and provides further evidence of the precise manner in which the *Diplolepis* females lay their eggs. Eggs of *D. rosaefolii* are normally laid on the upper three distal leaflets, but the reason for the almost total exclusion of the lower proximal leaflets is unclear. The distal leaflets may simply be more accessible to the ovipositing female. If the leaflets are still enclosed in the stipules or have just begun emerging from the stipules when the female oviposits, these leaves would be uppermost and therefore the first to emerge from the stipules. However, Declerck-Floate and Steeves (1995) found that the gall midge *Cystiphora sonchi* (Bremi) oviposits on the tips of the leaves of *Sonchus arvensis* L. because of the

tip to base pattern of leaf maturation. This type of development, known as basipetal development, is also present in the leaves of *R. virginiana* (LeBlanc unpublished data) and could explain the placement of eggs on the three distal most leaflets. At the time of oviposition, the three distal most leaflets would be more developed than the proximal leaflets and would be more accessible to the wasp because of their location on the apical meristem.

Rohfritsch (1992) lists a number of factors which are believed to be involved in cynipid gall initiation including i) tissue wounding during oviposition, ii) influence of the ovipositional fluid, and iii) activity of the larva. When the *D. rosaefolii* female lays an egg on the leaf, only one epidermal cell is damaged so one would question whether the subsequent response is elicited by this factor. However, the ovipositional fluid covers a number of epidermal cells and therefore it is more likely that this is the mediator involved in initiation. Rohfritsch (1992) reports that the ovipositional fluid acts as an interface between the egg and the underlying plant, allowing for the movement of substances between the two. This suggests that some influence may also be exerted by the unhatched larva through this interface. Even before the larva hatches, significant changes take place directly below the point of attachment of the egg. The initial signs of change in the leaf cells is a fragmentation of the vacuole in cells underlying the egg. This manifestation is believed to be the first stage in the autolytic process (Rohfritsch 1992). The opening that forms as a result of this lytic effect will become the chamber that the larva enters on hatching. Evidence of this autolytic process is visible as remains of lysed cells surrounding the chamber at the time of larval entry.

Hough (1952) found that the nutritive tissue in galls of *Neuroterus quercus-baccarum* L. on oak leaves differentiated in a dome surrounding the head of the horizontally oriented larva. In *D. rosaefolii* galls, nutritive tissue appeared to differentiate at one end of the chamber first and eventually spread out, surrounding the larval chamber. This may be the result of larval feeding patterns initially starting at one side of the chamber and spreading to other points as the larva turns while feeding. During early stages of gall development, the larval chamber is small in relation to the size of the larva and restricts its movements. The fact that nutritive tissue is sparse at the top and bottom of the chamber may result from the larva feeding less frequently in these areas as they are not easily accessible. Sclerification of the primary plates also exhibit polarity in differentiating, with the upper plate appearing before the lower plate. Reference to a polar differentiation of sclerenchyma has not been mentioned previously in the literature. Shorthouse (1975) found that the initial differentiation of the sclerenchyma tissue in four of the *Diplolepis* galls he studied involved a disc-shaped patch of cells above and below the chamber. In galls of *D. lens, D. nebulosa*, and *D. ignota* he found the discs were incorporated into the sclerenchymal layer as further differentiation occurred. In galls of *D. ignota* two circumscribing layers of sclerenchyma are present: the inner layer consisting of thin-walled cells while the outer layer contains thick-walled cells. The initial discs become incorporated into the inner layer. The sclerenchymal layers in *D. rosaefolii* galls are plate-like rather than circumscribing as seen in *D. ignota* galls and are devoid of discs. Furthermore, the cell walls appear to be thinner in the sclerenchyma of the secondary outer layer than in the sclerenchyma of the primary layer. Mani (1964)

described cynipid galls with two concentric layers of sclerenchymal tissue where the outer layer contained cells with thinner walls than those in the inner layer. These descriptions are consistent with those observed in *D. rosaefolii* galls. The types of lentil-shaped galls studied by Hough (1952) in *Neuroterus quercus-baccarum*, Schnetzler (1964) in *Neuroterus numismatis L.*, and Shorthouse (1975) in *D. lens* did not contain secondary sclerenchymal plates, although *Neuroterus quercus-baccarum* and *Neuroterus numismatis* galls have two discs of sclerenchyma in the upper gall wall but they do not extend out as much as the plates in *D. rosaefolii* galls.

During the maturation stage, lateral sclerenchyma differentiated from parenchyma at the edges of the gall chamber. Hough (1952) also observed lateral sclerenchyma in galls of *Neuroterus quercus-baccarum*, but these were very elongated cells and each cell extended from one sclerenchymal plate to the other. The lateral sclerenchyma in *D. rosaefolii* galls consists of a series of similar cells running from the upper to the lower plates. Fourcroy and Braun (1967) propose that the lateral sclerenchyma is a protective adaptation to avoid crushing of the galls when they drop to the ground. Because the *Neuroterus quercus-baccarum* gall is suspended from the lower surface of a leaf and drops off the leaf at maturity, it may require greater protection than the *D. rosaefolii* gall which is incorporated in the leaf tissue and therefore falls with the leaf during normal leaf fall in the autumn. However, the sclerenchyma could aid in protecting the larva against desiccation, decomposition, or act as an insulator while the gall overwinters in the leaf litter.

An increase in vascularization of the gall tissues was observed in a number of

galls at the maturation stage. This probably results from an increased demand on the nutritive tissue from the larva as its increasing size requires more sustenance. This increased demand would be provided by the vascular tissue. Rohfritsch (1992) found that larvae enter their most active feeding stage only after the gall tissues are differentiated. At the maturation stage the larvae would be in this active feeding stage in which they would consume a much larger volume of cells and therefore the additional vascularization would provide materials to replenish the consumed cells.

Initial changes in gall size occurred through growth and division of the cells in the parenchymal tissue as this is the only tissue present between the epidermal layers in the initiation stage. The amount of parenchyma continues to increase until the end of stage 3, adding bulk to the gall wall. Once the nutritive tissue differentiates during stage 2, it enlarges and contributes to increased gall size. However, the increase in size of the sclerenchyma cells which make up the primary and secondary plates accounts for most of the growth in the gall.

4.3 Changes in gall morphology induced by *Periclistus*

The initial change observed in the *D. rosaefolii* gall tissues when they become inhabited by *Periclistus* is the proliferation of a large amount of parenchyma at the sides of larval chambers. These cells are rectangular in cross section allowing them to be easily distinguished from the square cells induced by *D. rosaefolii*. It is unclear whether this initial change is induced by the larva or by the female either through a substance introduced into the gall during oviposition (e.g. growth promoting substance such as an

auxin) or through the ovipositional fluid. Because an inquiline gall may contain larvae and eggs at the same time, the proliferation of the parenchyma could be induced by the hatched larvae. Shorthouse (1975) observed similar proliferation of parenchyma in *Periclistus*-inhabited galls of *D. lens* but he did not speculate on the mode of induction.

The proliferation of the parenchyma extends predominantly to the upper wall as the gall develops further and results in the distinctive upraised central portion of *Periclistus*-inhabited galls visible on the outer surface of the leaf. This feature of the gall allows them to be easily distinguished from galls inhabited by *D. rosaefolii* larvae. At this same stage of development, cells lining the larval chamber (or chambers) in *Periclistus*-inhabited galls differentiate, forming the nutritive tissue. Cells of the nutritive tissue induced by *Periclistus* are rectangular in cross section and are approximately the same size throughout the tissue layer. This is in contrast to the nutritive cells in *D. rosaefolii* galls which are square in cross section and larger at the edge of the larval chamber. Furthermore, the larval chamber in *D. rosaefolii*-induced galls is lined with a relatively large amount of nutritive tissue at the sides of the gall but only a thin layer at the top and bottom. Shorthouse (1975; 1980) described changes in the amount and distribution of the nutritive cells in *Periclistus*-inhabited galls that were similar to the findings reported in this study.

In *Periclistus*-induced nutritive cells there appears to be more extensive vacuolation and a smaller amount of lipids than in the nutritive cells induced by *D. rosaefolii*. Furthermore, the distribution of starch is not the same in those two types of nutritive cells. In *Periclistus*-inhabited galls the starch granules are found in all cells of

the nutritive layer. However, in *D. rosaefolii*-induced galls starch is located in the parenchyma which adjoins the nutritive cells, and none is present in the nutritive cells. Bronner (1981) found similar cytological changes in inquiline modified galls of *Neuroterus quercus-baccarrum* L. on *Quercus pedunculata* Ehrh. and postulated that there is a relationship between increased vacuolation of the inquiline cells and the change in starch deposition. Bronner (1977) showed that the amount of starch in the nutritive tissue is related to the amount of stimulation the cells receive. Cells closest to the larval chamber are more stimulated to high physiological and metabolical activity due to larval feeding and they contain smaller vacuoles and no starch, while cells further away from the larva are less stimulated and contain starch in an increasing gradient toward the epidermal layer. Inquilines induce the proliferation of more nutritive tissue than the inducer, and therefore it would seem more appropriate to hypothesize that the cells containing increased numbers of vacuoles and starch would be more stimulated. Because starch is normally found in storage tissues in the plant, this may have prompted this author to consider it less stimulated. Further study in this area could help to gain a more thorough understanding of the differences between these two cells types.

Changes in sclerenchymal layer patterns are dependent on the stage of gall development at which the inquiline eggs are laid in the gall chamber. Shorthouse (1975) only observed *Periclistus* eggs in galls in which no sclerenchymal layer was present. This suggests that *Periclistus* species which are inquilines in *D. rosaefolii* galls have adapted to enable them to oviposit in galls at later stages of development than is possible for *Periclistus* species inhabiting other galls. This may be because *Periclistus* adults emerge

at a time when few or no young *D. rosaefolii* galls are present in which the *Periclistus* females can oviposit.

The *Periclistus*-induced sclerenchyma cells are similar to the newly differentiated sclerenchyma of the plates in the *D. rosaefolii* gall. Irregularities of the upper sclerenchymal plate are much more pronounced and are present at a much earlier stage of development in *Periclistus* galls than in those inhabited by *D. rosaefolii*. No reference to the presence of an undulating sclerenchymal layer could be found in the literature and it is unclear what significance this may have in the development of the gall. If the sclerenchymal layer serves a mechanical role, as has been suggested by a number of authors (e.g. Mani 1964; Shorthouse 1975; Rohfritsch 1992), then these undulations may provide greater strength and flexibility than a similar layer with no undulations. It is interesting to note that *D. rosaefolii* galls inhabited by *Periclistus* at early stages of gall development have only one sclerenchymal plate and not two sets of plates as is found in the *D. rosaefolii* galls. It may be that the single plate with undulations in the adaxial wall of the *Periclistus* gall provides a similar amount of support as the double plate of fully mature *D. rosaefolii* galls but with a decreased time expenditure to the inquiline larva. Because *Periclistus* inhabits the galls at a later time in the growing season, it may have developed this structural adaptation to hasten the developmental process so that galls would reach maturity at a faster rate than is seen in *D. rosaefolii* galls. It would be interesting to study inquiline-modified galls in more southern climates to see whether this structural adaptation persisted in areas with longer growing seasons.

The large increase in cellular mass which is induced by *Periclistus* precludes the

need for more extensive vascularization of the gall tissues to develop and maintain this increase. Bronner (1981) noted enhanced vascularization in galls of *Pediaspis aceris* on maple leaves when inhabited by the inquiline *Dichatomus acerinus* (Mandl).With the increase in the number of larvae in a gall which is often the case in inquiline-inhabited galls, the nutritional requirements of the extra larvae would also contribute to the need for more vascularization in order to fulfill their feeding requirements.

4.4 Summary

Galls of *D. rosaefolii* provide a useful model for studying the development of a leaf gall. Although the galls are similar in development to those that have already been studied, a number of differences are observed in the types of tissues and their arrangement in the gall. The presence of the double sclerenchymal layer is unique in lenticular galls and the lack of vascularization in the central portions of the maturing gall wall has not been reported in any cynipid galls studied to date. These differences suggest that tissue development in galls is much more dynamic than can be accommodated in a single model. The few lenticular galls that have been studied have many similarities in their tissues. A number of events in the development of lenticular galls diverge from Rohfritsch's (1992) model, which is based on development studies in circular galls. Therefore, as more lentil-shaped galls are studied it may be necessary to provide a new or modified model with this specific shape.

Few studies contain information on galls inhabited by an inquiline which has modified the gall tissues. A number of changes are brought about by the presence of the

Periclistus larva in the galls induced by *D. rosaefolii*. These include changes in the shape and abundance of nutritive tissue, the arrangement and in some cases the presence of the double sclerenchymal layers, and the shape and abundance of the parenchymal tissue. Furthermore, the epidermal cells and the parenchyma cells of the outer gall wall have characteristics of cells observed in the ungalled leaf tissue (such as the presence of numerous chloroplasts in the inquiline-modified galls); these features are not present in the gall when it is inhabited by *D. rosaefolii*. Most inquilines are closely related taxonomically to the gall-inducing insect and it is therefore interesting to observe the extent of changes brought on by the inquiline (Shorthouse 1973; Ronquist 1994). Due to the close taxonomic relationship between most inquiline and inducer insects, changes induced by the inquiline support the belief that gall morphology is determined by both the insect and plant phenotype. If plant phenotype was the sole influence on gall morphology, the inquiline-inhabited gall would be the same as that produced by the inducer. The morphology of *Periclistus*-modified galls in the present study more closely parallels the cynipid model of development proposed by Rohfritsch (1992) than does the inducer-inhabited gall. From this we can postulate that if *Periclistus* was evolving toward the ability to induce its own galls, these galls would be more circular than lenticular and would contain concentric rings of tissue surrounding the larval chamber as is found in the majority of cynipid galls.

Further study of *D. rosaefolii* and its inquiline-modified galls is required to provide information on the exact timing of the developmental stages in the galls. This would best be carried out in the laboratory and might provide insight into the initial

inducing mechanism in both of these galls. More study is required in this area if we are ever to determine the mechanism which initiates and maintains the development of the gall tissues. Only when this mechanism is determined can we hope to use this model to further our knowledge and use of the morphogenetic potential which is exemplified by galls on plants.

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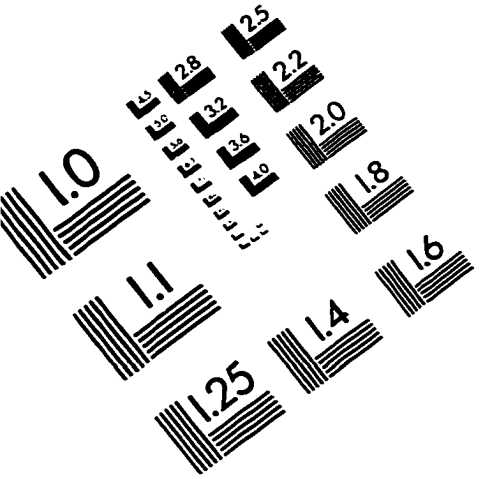
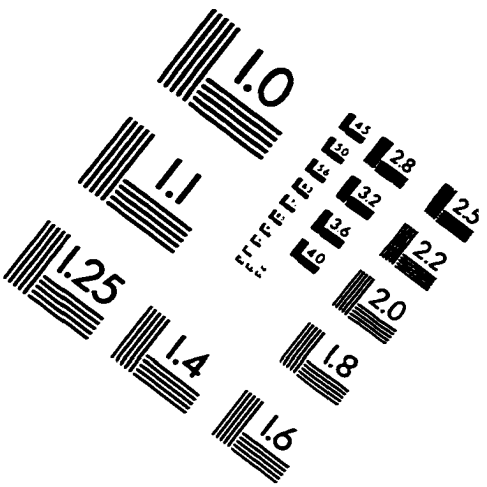
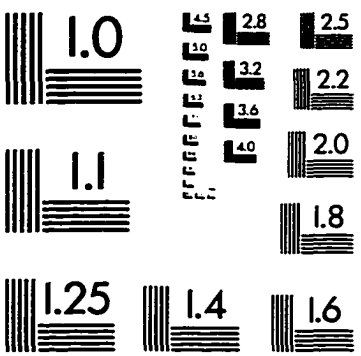
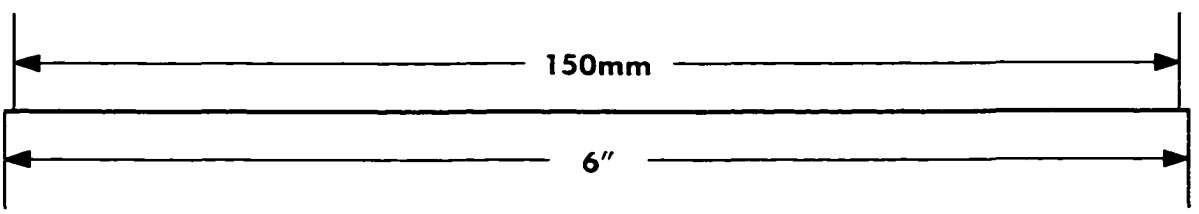
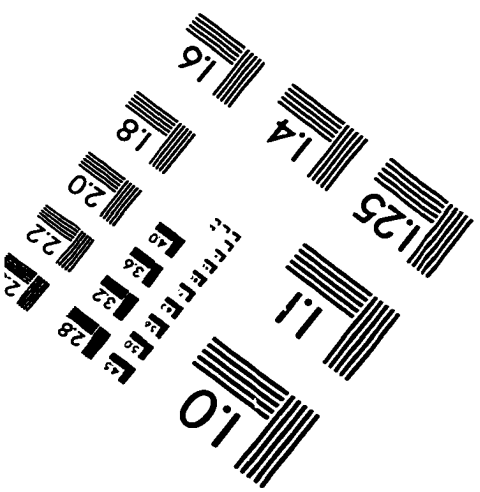
IMAGE EVALUATION
TEST TARGET (QA-3)

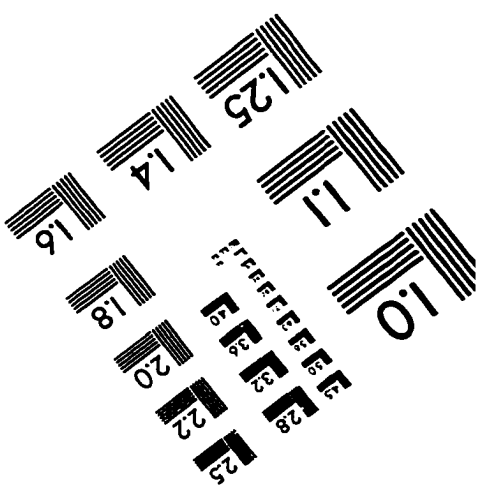












APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989