

Chapter 3. MORPHOLOGY OF SMALL BROOMRAPE (*Orobanche minor* SM.) ATTACHMENTS ON RED CLOVER (*Trifolium pratense* L.)

3.1 INTRODUCTION

Small broomrape (*Orobanche minor* Sm.), also known as clover broomrape, is an achlorophyllous holoparasite that subsists on the roots of a variety of the leguminous crops from which it derives carbon, water, and nutrients needed for further growth. *O. minor*, along with other species of genus *Orobanche*, are devastating parasites of a number of economically important plants, including broad bean (*Vicia faba* L.), pea (*Pisum sativum* L.), alfalfa (*Medicago sativa* L.), red clover (*Trifolium pratense* L.), white clover (*Trifolium repens* L.), and some varieties of vetch (*Vicia* spp.). Broomrapes lack chlorophyll, and thus lack the ability to synthesize own food (Baccarini and Melandri 1967; Saghir et al. 1973). Broomrape growth occurs at the expense of water, minerals, and organic compounds obtained from the host plant, which consequently leads to host plant exhaustion, lower biomass accumulation (Baker et al. 1995), and complete crop failure (Foy et al. 1989).

O. minor produces fine and almost dust-like seeds in large quantities (Kadry and Tewfic 1956). Broomrape seeds remain dormant and do not germinate until they are near a suitable host and the environmental conditions are favorable. In order to germinate, *Orobanche* spp. seeds must meet two basic requirements (Foy et al. 1989). Broomrapes must undergo a period of seed preconditioning in a suitable environment (Jain and Foy 1987; Kasasian 1973), and require presence of a germination stimulant, a chemical signal from host root exudates, that 'triggers' the germination of preconditioned seeds (Foy et al. 1989). As broomrape seeds germinate, they develop an elongated radicle. If a germinating broomrape seed is within reach of a host root, its radicle can attach to the root surface. Although the natural germination stimulant(s) for *Orobanche* spp. has(have) not been found, some broomrapes respond well to artificial plant growth stimulants such as strigol, its analogs, GR-7 and GR-24, and others (Abdel Halim et al. 1975; Johnson et al. 1976; Saghir 1986; Stewart and Press 1990; Zahran 1982).

After the attachment to the host root, the radicle undergoes rapid cell division, thickens, and forms a haustorium, which penetrates the root cortex, and inhibits the vascular system of the host. The portion of the parasite, which remains outside of the root tissue transforms into a tubercle (Foy et al. 1989; Parker and Riches 1993). In turn, tubercle develops vestigial roots and produces a shoot that emerges above the soil surface and forms a floral spike. After flowering, capsules are formed containing numerous seeds, which are later dispersed by wind, water, and/or human activity (Foy et al. 1989; Sauerborn 1991).

O. minor attachments are often accompanied by a formation of a characteristic swelling of the host plant root tissue around the point of parasite penetration. Literature reports indicated a correlation between parasitic weeds infestations and rhizobacterial nodulation in legume crops (Parker and Riches 1993). In addition, bacterial nodulation was assumed to be the mechanism by which broomrape could by-pass the root rhizodermis and gain infestation (Figure 3.1.; Petzoldt 1979). Similarly, *O. minor* attachments on legumes were speculated to involve rhizobacterial nodules as points of intrusion. Schmitt et al. (1979) concluded that *Orobanche* infestation does not occur before formation of legume nodules on the host plant. Moreover, the infestations are more intense in aerobic conditions (Cubero 1973) when more nodules are produced. However, these reports are yet to be confirmed by others.

Legumes have a unique ability to form symbiotic relationship with the diazotrophic bacteria belonging to family Rhizobiaceae. The host legume provides bacteria with an ecological niche in nodules, specialized structures formed on the roots of the host upon inoculation with rhizobacteria (Hirsch 1992; Vance et al. 1988).

Rhizobacteria enter the root hairs of a susceptible host legume through a series of complex interactions, which begin with a chemical signal exchange between the host and bacteria (Franssen et al. 1992; Hirsch 1992), which is followed by the physical contact between rhizobia and the surface of the root hair. Adhesion of bacteria is followed by deformation of the root hair resulting in the characteristic shepherd's crook appearance (Pawlowski and Bisseling 1996; Somasegaran and Hoben 1994). The hyphae-like infection thread develops gradually in the root hair as an invagination of the root hair wall (Berry and Sunell 1990). The infection thread contains large numbers of rhizobial cells, as it ramifies through the root cortex toward developing nodule primordium (Callaham and Torrey 1981; Somasegaran and Hoben 1994; van Spronsen et al. 1994). Other root cells become infected, continue to divide and later form the nodule where nitrogen fixation takes place.

In our previous research we observed an increase in *O. minor* infestations of red clover upon inoculation with rhizobacteria (data not shown), indicating a relationship between *O. minor* parasitism and rhizobacterial nodulation in legumes. This study was directed to analyze the morphology and anatomy of *O. minor* attachments on red clover and is complementary to our research on the correlation between rhizobacterial nodulation and *O. minor* parasitism.

3.2 MATERIALS AND METHODS

3.2.1 PLANT MATERIAL

Seeds of small broomrape (*O. minor* Sm.) were provided by Dr. R. Eplee¹, from the stock collected in Thomas Co., GA, USA. *Orobanch*e spp. seeds were stored at 25 C in the dark in sealed containers. Red clover seed mix was purchased from the Blacksburg Feed and Seed Co². Air-dried red clover seeds were refrigerated at 4 C in a plastic bag until used. Inoculum culture, *Rhizobium leguminosarum* bv. *trifolii*, was purchased from the American Type Culture Collection³ and cold-stored at 4 C before use. According to USDA APHIS⁴, *Orobanch*e spp. are considered to be noxious weeds in the United States; thus, handling of the broomrape material was conducted in strict quarantine.

3.2.1.1 *Orobanch*e minor Seed Preparation

O. minor seeds were surface-disinfected for 15 min in a mixture of 1% sodium hypochlorite (commercial bleach) and 1% v/v Tween 20⁵ following a 15-s immersion in 70% ethyl alcohol⁶ (by vol). Subsequently, seeds were rinsed in autoclaved⁷ distilled water three times, 15 min. each time. Surface-cleaned *O. minor* seeds were placed in a 9-cm-diam Petri dishes⁸ between two glass-fiber filter paper (GFFP)⁹ disks. Sealed with parafilm¹⁰, Petri dishes were stored at 25 C in the dark.

3.2.1.2 Red Clover Seed Preparation, Inoculation, and Planting

Red clover seeds were surface-disinfected, as described for broomrape seeds. A batch of air-dried, surface-disinfected red clover seeds was planted immediately in plastic trays¹¹ in autoclaved vermiculite¹², which was used as a soil substitute. A second batch of red clover seeds (10 g) was inoculated with rhizobium-containing peat powder¹³, prior to being planted in autoclaved vermiculite. Rhizobium-containing peat powder was prepared from yeast-mannitol broth (YMB), *Rhizobium leguminosarum* bv. *trifolii* culture, and autoclaved peat powder. YMB constituents included a mixture of K₂HPO₄¹⁴ (0.5 g L⁻¹), MgSO₄ x 7H₂O¹⁵ (0.2 g L⁻¹), NaCl¹⁶ (0.1g L⁻¹), dissolved in distilled (autoclaved) water (1.0 L), and followed by the addition of the D-mannitol¹⁷ (10 g L⁻¹) and yeast extract¹⁸ (0.5 g L⁻¹). While the mixture was continuously agitated,

the pH of the liquid was adjusted to 6.8 with NaOH. Once the desired pH was achieved, the mixture was autoclaved at 121 C for 15 min. YMB was allowed to cool, followed by the inoculation with *Rhizobium leguminosarum* bv. *trifolii*, and left on a shaker at 25 C for 36 h. Peat powder was prepared from commercially available alfalfa-clover inoculant¹⁹. A batch of 80 g of fresh inoculant (21.5% moisture content) was dried in an oven²⁰ for 36 h. Two autoclaved jars²¹ were filled with 25 g of dried peat and autoclaved for 50 min each at 121 C, followed by an addition of 15 ml per jar of YMB pre-inoculated with rhizobacteria. Jars with inoculated peat powder (35% moisture content) were sealed and placed in the dark for two weeks to allow rhizobia grow and develop.

A two-step procedure, described by Somasegaran and Hoben (1994), was used to inoculate red clover seeds. The sticker (3 ml, 20% sucrose²² solution) and the semi-powdered inoculant (0.5 g peat powder; 7×10^4 rhizobia cells/g of inoculated red clover seed)²³ were applied to the red clover seeds separately. In the first step, the seeds were uniformly coated with the sticker solution. In the second step, the semi-powdered inoculant was added to the sticky seeds.

Inoculated red clover seeds were planted in autoclaved vermiculite in separate plastic trays and, along with the trays containing non-inoculated red clover, were placed in the controlled environment room under fluorescent lights. Light intensity was $100 \mu\text{mol m}^{-2} \text{s}^{-1}$, with the temperature maintained at 24 C during the 12-h light period and 20 C during the 12-h dark period. Half-strength Hoagland's nutrient solution²⁴ (Hoagland and Arnon 1950) was added to the non-inoculated flats throughout seed germination and plant establishment. Simultaneously, inoculated plants received half-strength Hoagland's solution that did not contain nitrogen (-N), in order to stimulate nodulation.

3.2.2 POLYETHYLENE (PE) BAG METHOD

Healthy, 20-d-old, red clover plants were transplanted into polyethylene (PE) plastic bags²⁵. Red clover plants inoculated with *Rhizobium leguminosarum* bv. *trifolii* were grown separately from non-inoculated plants. *O. minor* was grown on red clover for 39 d in the PE bags (15 x 30 cm), each containing glass fiber (GF/A)²⁶ filter paper sheet (14 x 23 cm), according to Parker and Dixon (1983). Red clover plants, two per bag, were placed at the top of the bag on GF/A filter paper, previously moistened with 10 ml of sterile distilled water, using a disposable syringe. The red clover root system was spread on the filter paper to cover the largest area possible. At the same time, approximately 5 to 6 cm from the top of the bag, *O. minor* seeds (0.015 g) were uniformly dispersed in an 80-cm² area where red clover root growth was anticipated. The top of the bag was folded over a wooden dowel and stapled on both sides.

Each bag received half-strength Hoagland's nutrient solution (at least 20 ml per bag) with inoculated plants receiving a (-N) mixture. PE bags were hung in the cardboard boxes with only red clover foliage exposed to the light. Boxes were placed in the controlled environment room with identical conditions described for red clover planting, with fluorescent lights placed approximately 22.5 cm above red clover foliage. Throughout the growth period, plants were watered as required with half-strength Hoagland's solutions (-N for inoculated plants; normal for non-inoculated plants) injected into the bags through a small opening cut on the side of each bag.

3.2.3 ASEPTIC METHOD

Sterile 100-ml glass jars were filled to 1/3 of the volume with 10 g of dry autoclaved vermiculite. Two spoonfuls of surface-cleaned *O. minor* seeds (0.015 g per spoon) were placed in the jars, and mixed, using sterile spatula, to ensure even distribution of *O. minor* seeds in autoclaved vermiculite. Ten surface-sterile red clover seeds were introduced directly on the top of the vermiculite, and placed around the perimeter and in the center of the jars.

A separate batch of surface-sterilized red clover seeds was inoculated with aseptically prepared rhizobium-containing peat powder, and placed in the aseptic jars similarly to the non-inoculated plants, as described above.

Each jar received autoclaved half-strength Hoagland's solution (10 ml/jar) with inoculated plants receiving a (-N) mixture. All jars, covered with clear plastic caps and sealed with parafilm, were placed in the controlled environment room under fluorescent lights positioned approximately 45 cm above the jars. Light intensity was $100 \mu\text{mol m}^{-2} \text{s}^{-1}$, with the temperature maintained at 24 C during the 12-h light period and 20 C during the 12-h dark period. Plants were grown for 45 d. Through the growing period selected jars were sampled randomly. After 45 d red clover plants were collected and numbers of *O.minor* attachments per plant were recorded. All data were subjected to TTEST model analysis, using SAS statistical software (SAS 1989).

3.2.4 EXAMINING NODULES AND BROOMRAPE ATTACHMENTS

Observations of the plant material were made using a Leica® stereomicroscope²⁷, Scanning Electron Microscope²⁸ (SEM), and Zeiss® stereomicroscope²⁹. Pictures were taken with the 35-mm camera mounted on the top of the stereomicroscopes, and with Polaroid® camera mounted on the SEM. Kodak® Ektachrome color slide film³⁰ was used in the 35-mm cameras.

Tissue fixation for SEM was conducted according to the standard manual for SEM tissue preparation, and performed by the laboratory specialist (PPWS Electron Microscopy Facility, Virginia Tech) under supervision of Dr. R. L. Grayson³¹. For microtome sectioning, tissues were prepared according to the procedures suggested by Dr. O. K. Miller³² (Dept. Biology, Virginia Tech), described in more detail below.

3.2.4.1 Preparation, Dehydration, and Infiltration of Plant Material

Randomly selected red clover plants, from aseptic jars, were collected to observe *O. minor* attachments and nodules formed. Samples of attachments and nodules from PE bags and aseptic jars were excised from the roots, trimmed to the desired size, and fixed in FAA fixative. FAA fixative was prepared from ethyl alcohol, glacial acetic acid³³, and formaldehyde diluted in water (50:5:10:35 by vol, respectively). All traces of vermiculite from the jar samples were removed to prevent possible damage to the microtome³⁴ knife and to reduce the amount of foreign materials that could appear in the sections to be observed under the microscope. Plant material to be sectioned was prepared through a series of dehydration and infiltration steps outlined in Table 3.1, through which tissues were infiltrated with paraffin oil³⁵ and placed in paraplast³⁶, melted in an oven³⁷ at 60 C.

3.2.4.2 Embedding, Sectioning, and Mounting of the Plant Material

Dehydration series were followed by the embedding of the plant tissues into paraffin molds³⁸ using the warmplate³⁹. The bottom of the mold was filled with paraplast, and the specimen was positioned in the desired orientation. After cooling to room temperature, the blocks were taken out of the mold. The solidified paraplast blocks were trimmed to within 0.5 cm of the specimen.

Sectioning was conducted using the microtome. A vessel of distilled water was prepared on the warmplate, with the temperature of the water maintained between 40 and 50 C. As the microtome knife moved across the paraffin block, the sections were removed from the blade with forceps, and immediately placed in the warm water. A 10 to 20-s period was allowed for the material to stretch out, and as the water cooled, the sections solidified in a stretched-out form.

Hardened sections were placed on glass slides⁴⁰, coated with a thin layer of egg albumin⁴¹. Egg albumin was prepared by mixing dry powder with a few drops of water until a homogenous mixture was obtained. Slides with mounted sections were allowed to dry at the room

temperature.

Prepared slides were dipped in three successive solutions of xylene⁴² (5 min each time), followed by dehydration in ethyl alcohol (5 min). The excess moisture was blotted off, and a drop of Permount mounting medium⁴³ was placed on the top of the sections, immediately followed by covering them with the cover glass. Prepared slides were examined under the Zeiss® stereomicroscope.

3.3 RESULTS AND DISCUSSION

Nodules and tubercles differed in coloration as seen in plant material sections. Active N-fixing nodules contained *leghemoglobin* as observed by their characteristic pink or brownish coloration in nitrogen-fixation zone of the nodules (Figure 3.2.). In contrast, tubercles of *O. minor* were uniformly brown to rust color on the exterior and a light yellow-brown inside (Figure 3.3.).

Whether red clover plants were inoculated or not with rhizobium, *O. minor* attachments were accompanied by the formation of a characteristic swelling of the root tissue around the point of parasite penetration (Figure 3.4.) which was not present at the initial stages (Figure 3.5.). The swellings were usually detectable within two days from the initial contact between *O. minor* radicle and red clover root. Furthermore, *O. minor* attachments did not appear to be situated over the distinct and developed nodules (Figure 3.6.). Not only did the swelling beneath the tubercle of the parasite have a different morphological appearance from a typical red clover nodule, but it also often contained splits of the root cortex (Figure 3.7.) uncharacteristic to the nodulation. The SEM of the nodules and *O. minor* attachments also demonstrated the difference between nodules and *O. minor* attachment anchoring on the host root (Figure 3.8). While nodules had a pointed place of association with the host root, *O. minor* attachments induced swellings that were occupying at least a portion of the red clover root.

O. minor attachments observed in the aseptic tests were similar to those developed on the red clover roots in PE bags, with nearly identical swellings formed beneath *O. minor* tubercles. However, in the jars containing non-inoculated plants the number of attachments was significantly less compared to the inoculated plants (Figure 3.9.). No spontaneous nodulation was observed in the non-inoculated treatments in both PE and aseptic tests. *O. minor* is a well known red clover parasite, and perhaps could attach without regard for the presence or absence of the rhizobacteria. However, absence of the attachments in other jars raises questions whether other factors beside germinating stimulants from the host root exudates and moist surroundings are important for the successful *O. minor* development. In aseptic jars red clover plants grew no

more than 3.5 to 4 cm high compare to 10 to 15 cm red clover plants grown in PE bags. Sealed in the moist environment of the jars, slow host growth could have affected the release of root exudates in the vermiculite.

Longitudinal sections of the parasitic attachments revealed an intrusion of *O. minor* haustorium through the cells of the root cortex (Figure 3.10.). It seems that the parasite invasion is accompanied by host root tissue proliferation around the penetrating haustorium (Figure 3.10.). Perhaps, this host tissue enlargement is what has been perceived as a developing nodule (Petzoldt 1979; Schmitt et al. 1979). Nevertheless, additional testing is necessary in order to fully understand the role of rhizobacterial involvement in *O. minor* parasitism.

3.4 CONCLUSIONS

As a part of an ongoing research on broomrape parasitism, experiments were conducted to observe the morphology of *O. minor* attachments on red clover. Parasitic attachments in the host plants inoculated with *Rhizobium leguminosarum* bv. *trifolii* were similar to those formed on the non-inoculated plants. The parasite attachments were accompanied by swelling of host root tissue at the point of parasite penetration. Microscopic observations indicated that the enlargement of the host root tissue could be the result of parasite intrusion, root cortex enlargement, or both. Although there were no attachments found to be situated over distinct and mature nodules, addition of rhizobia could play a role other than as a preferential point of parasite entry, such as influencing the host-parasite signal exchange.

Further research is needed on the host-parasite interactions involving rhizobacterial nodulation. Hopefully, the current research will aid in the future study of the biology and physiology of broomrape parasitism.

3.5 SOURCES OF MATERIALS

- ¹ Dr. R. Eplee, Oxford Plant Protection Center, USDA-APHIS-PPQ, 901 Hillsboro St., Oxford, NC 27565.
- ² Red clover seeds, commercial mix. Blacksburg Feed and Seed Co., 1212 N Main St., Blacksburg, VA 24060.
- ³ Inoculum culture. *Rhizobium leguminosarum* bv. *trifolii*, cat. # 14479; American Type Culture Collection ®, 12301 Parklawn Drive, Rockville, MD 20852.
- ⁴ USDA APHIS. Executive Branch of the US Government: United States Department of Agriculture: Animal and Plant Health Inspection Service.
- ⁵ Tween 20 ®, polyoxyethylene sorbitan monolaurate, a surfactant. ICN Biomedicals, Inc., 1263 South Chillicote Road, Aurora, OH 44202.
- ⁶ Ethyl alcohol, 200 proof. Aaper Alcohol and Chemical Co., Shelbyville, KY 40065.
- ⁷ Thermatic 60 autoclave. Castle Sybron, 1777 E. Henrietta Rd., Rochester, NY 14602.
- ⁸ Plastic Petri dish (08-757-12). Fischer Scientific Co., 711 Forbes Ave., Pittsburgh, PA 15219.
- ⁹ Glass-fiber filter paper (GFFP). Fischer Scientific Co., 711 Forbes Ave., Pittsburgh, PA 15219.
- ¹⁰ Parafilm M, laboratory film. American National Can, Greenwich, CT 06830.
- ¹¹ Plastic trays. 2 qt rectangle Rubbermaid food storage containers; cat. 42733WX; Consolidated Plastics Co. Inc., 8181 Darrow Road, Twinsburg, OH 44087.
- ¹² Vermiculate. Wetsel Seed Co., PO Box 791, Harrisonburg, VA 22801.
- ¹³ Rhizobium-containing peat powder. Prepared according to Somasegaran and Hoben (1994) using yeast-mannitol broth (YMB).
- ¹⁴ Potassium phosphate dibasic powder (3254). J. T. baker Chemical Co., Phillipsburg, NJ.
- ¹⁵ Magnesium sulfate crystal (M-63). Fischer Scientific Co., 711 Forbes Ave., Pittsburgh, PA 15219.
- ¹⁶ Sodium chloride (S-9625). Sigma Chemical Co., PO Box 14508, St. Louis, MO 63178.
- ¹⁷ D-mannitol (Mannite, M-1902). Sigma Chemical Co., PO Box 14508, St. Louis, MO 63178.
- ¹⁸ Yeast extract (Y-0500). Sigma Chemical Co., PO Box 14508, St. Louis, MO 63178.
- ¹⁹ Alfalfa-clover inoculant (nitrogen fixing bacteria) peat powder (5 oz. pack). Southern States Cooperative, Richmond, VA 23230.
- ²⁰ Stabil-Therm (OV-500C; laboratory oven). Blue M Electric Co., Blue Island, IL 60406.
- ²¹ Vessels for plant tissue culture (baby food jars) (V8630; 100 ml). Sigma Chemical Co., PO Box 14508, St. Louis, MO 63178.
- ²² Sucrose. Sigma Chemical Co., PO Box 14508, St. Louis, MO 63178.
- ²³ Rhizobia counts in the peat powder are approximate values, based on inoculant of 1.4×10^7 rhizobacterial cells / g of peat powder depositing approximately 87.5 cells / inoculated

red clover seed.

- ²⁴ Half-strength Hoagland's nutrient solution. Prepared in the laboratory according to the Hoagland and Arnon (1950).
- ²⁵ Polyethylene (PE) plastic bags. Fischer Scientific Co., 711 Forbes Ave., Pittsburgh, PA 15219.
- ²⁶ Glass-fiber filter paper (GF/A). Whatman International, Maidstone, UK, ME16 0LS.
- ²⁷ Leica ®GZ7 Stereomicroscope. Leica Inc., PO Box 123, Buffalo, NY 14240-0123.
- ²⁸ Scanning Electron Microscope 505 (SEM). Philips Electronic Instruments, Inc. (A North American Philips Co.), 85 McKee Drive, Mahwah, NJ. 07430.
- ²⁹ Zeiss® 872 E Axioscop. Carl Zeiss, 7082 Oberkochen, W. Germany.
- ³⁰ Kodak® Ektachrome color film (120 9485). Eastman Kodak Co., Rochester, NY 14650.
- ³¹ Dr. R. L. Grayson. 101 G Price Hall, Dept. PPWS, Virginia Tech. VA 24061-0331.
- ³² Dr. O. K. Miller. 2011 Derring Hall, Dept. Biology, Virginia Tech. VA 24061-0406.
- ³³ Glacial acetic acid (A38-212). Fischer Scientific Co., 711 Forbes Ave., Pittsburgh, PA 15219.
- ³⁴ Microtome 820. Spencer, American Optical Co., Buffalo 15. NY .
- ³⁵ Paraffin oil (O-119). Fischer Scientific Co., 711 Forbes Ave., Pittsburgh, PA 15219.
- ³⁶ Paraplast (Paraffin), tissue embedding medium. HRI 8889-502004. Lancer (Div. Of Sherwood Medical), a Brunswick Co., St. Louis. MO 63103.
- ³⁷ Stabil-Therm®, dry type biological incubator. Blue M 100A, Blue Island, IL 60406.
- ³⁸ Paraffin molds (Tissue embedding rings). Fischer Scientific Co., 711 Forbes Ave., Pittsburgh, PA 15219.
- ³⁹ Slide warmer. Chicago Surgical and Electrical Co., Medical Park. IL.
- ⁴⁰ Precleaned microscopic slides (72x25 mm; 12-550). Fischer Scientific Co., 711 Forbes Ave., Pittsburgh, PA 15219.
- ⁴¹ Egg Albumin (0255-15; Bacto E66 Albumen; Soluble). Difco Laboratories, P.O.Box 331058 Detroit, MI 48232-7058.
- ⁴² Xylene (UN 1307; Histological grade). Fischer Scientific Co., 711 Forbes Ave., Pittsburgh, PA 15219.
- ⁴³ Permout mounting medium (SP15-100), histological mounting medium. Fischer Scientific Co., 711 Forbes Ave., Pittsburgh, PA 15219.

3.6 LITERATURE CITED

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- features? *Plant Cell*. 8: 1899-1913.
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Table 3.1. Dehydration and infiltration steps in tissue preparation for microtomy^a

Step	Solution / Media	Concentration (by vol., respectively)	Time / Duration (h)
1	ETOH / H ₂ O (optional)	30 / 70	2
2	ETOH / H ₂ O / TBA	45 / 50 / 5	2
3	ETOH / H ₂ O / TBA	50 / 30 / 20	2
4	ETOH / H ₂ O / TBA	50 / 20 / 30	2
5	ETOH / H ₂ O / TBA	50 / 10 / 40	2
6	ETOH / TBA	50 / 50	2
7	ETOH / TBA	25 / 75	16
8	TBA	100	2
9	TBA	100	16
10	TBA	100	16
11	TBA / Paraffin oil	50 / 50	24
12	Paraffin oil	100	24
13	Paraffin oil / Paraplast	50 / 50	24
14	Paraplast	100	24
15	Paraplast	100	24
16	Paraplast (optional)	100	24

^aThe dehydration and infiltration steps were based on a minimum amount of time. Consistently poor sectioning results would suggest increasing exposure to the dehydration chemicals.

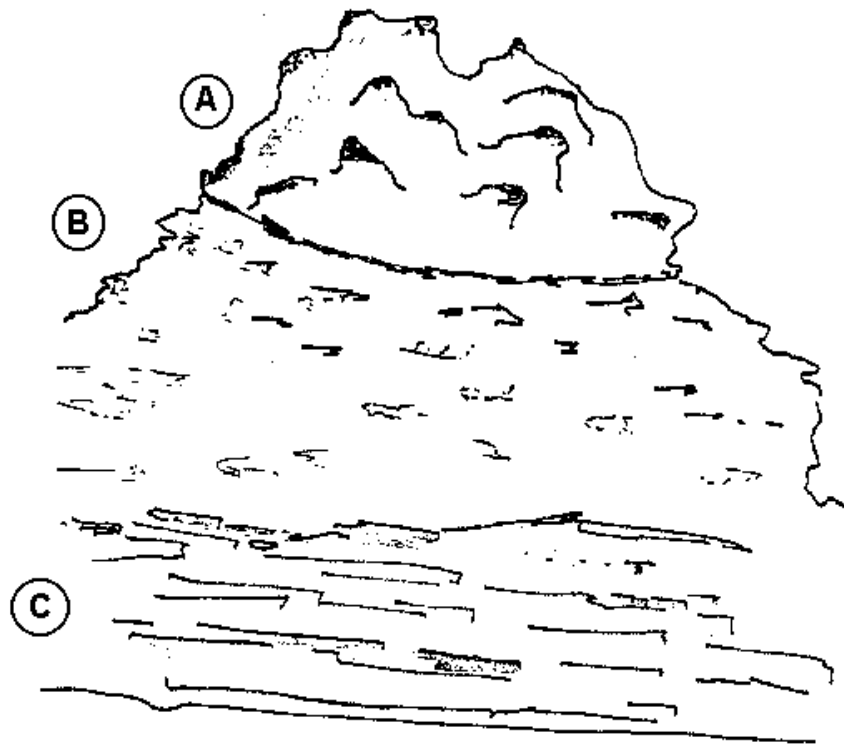


Figure 3.1. Initial haustorium of *O. crenata* situated on a bacterial nodule. A - haustorium of *O. crenata*; B - bacterial nodule; C - root of *Vicia faba*. (Petzoldt 1979).

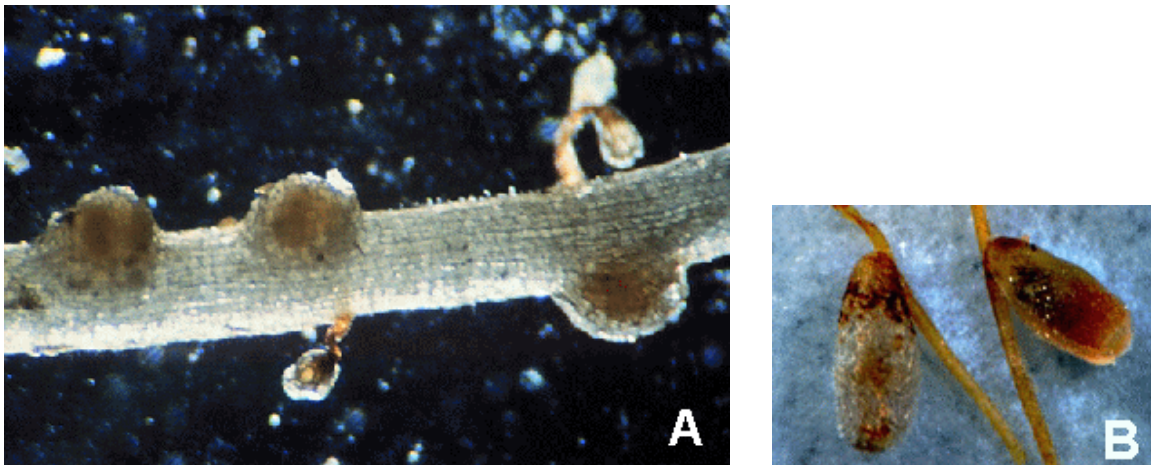


Figure 3.2. Rhizobacterial nodules formed on red clover. A – exterior of developing nodules; B – longitudinal section of a mature nodule

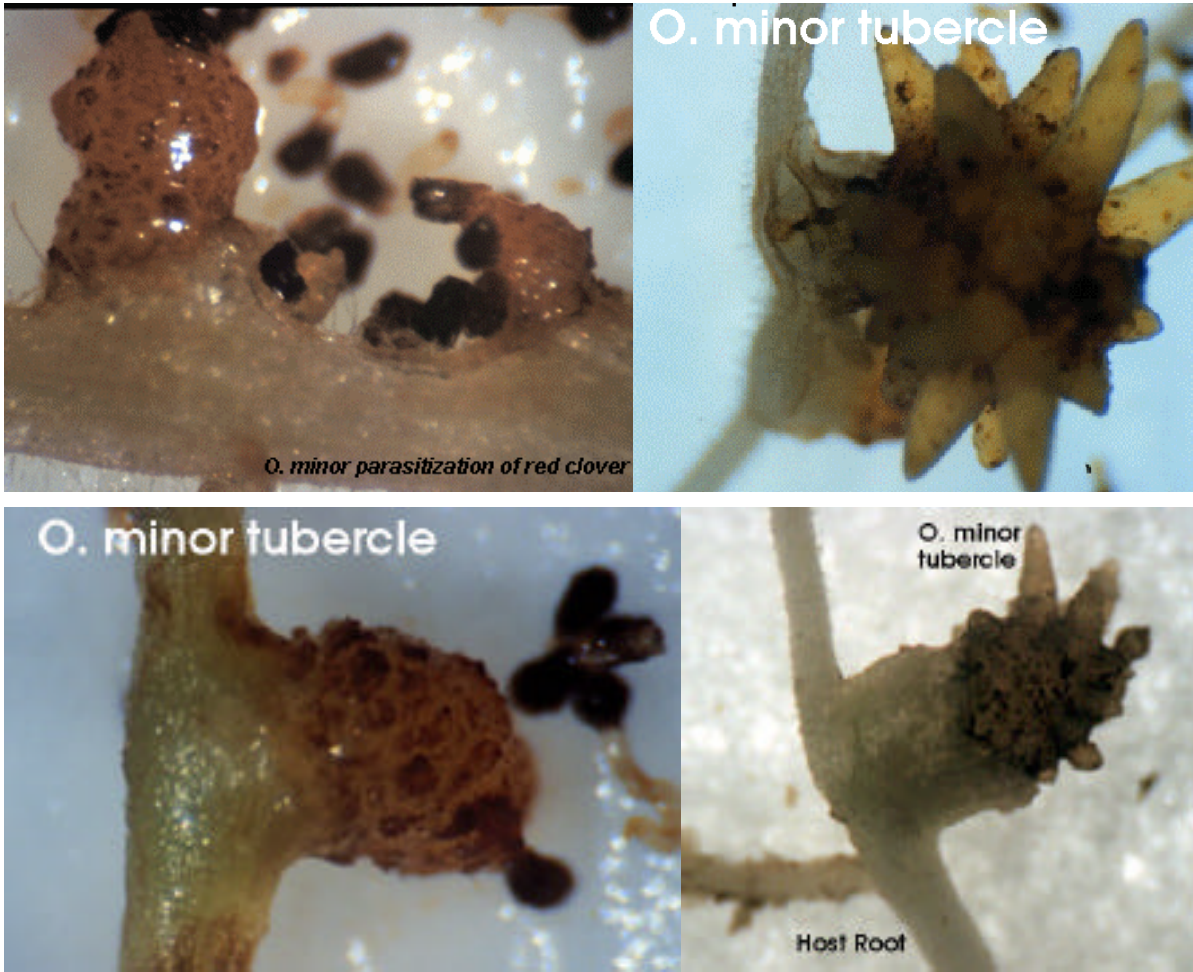


Figure 3.3. Small broomrape (*O. minor*) tubercles formed on the red clover roots.

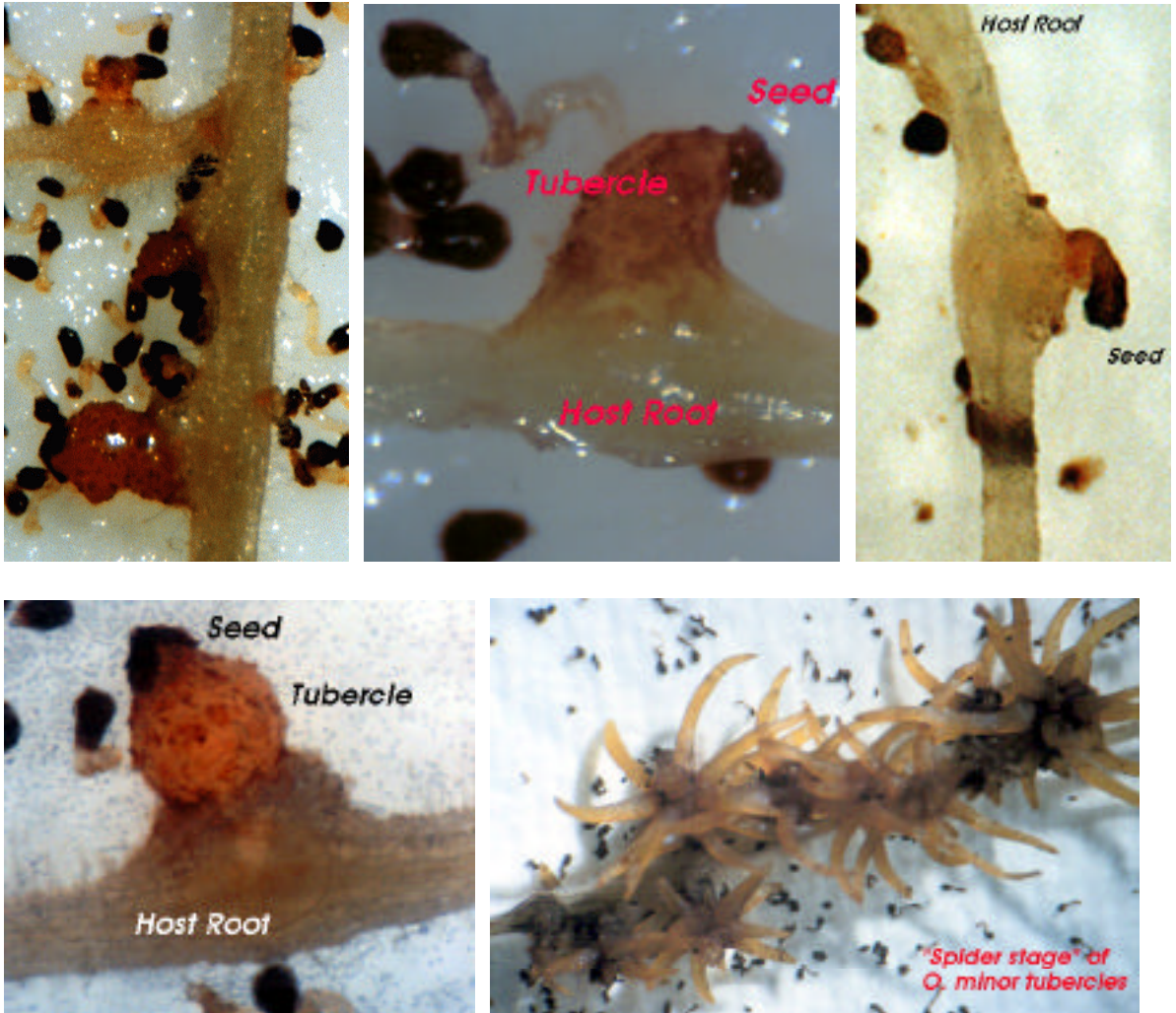


Figure 3.4. Small broomrape (*O. minor*) attachments formed on red clover roots.

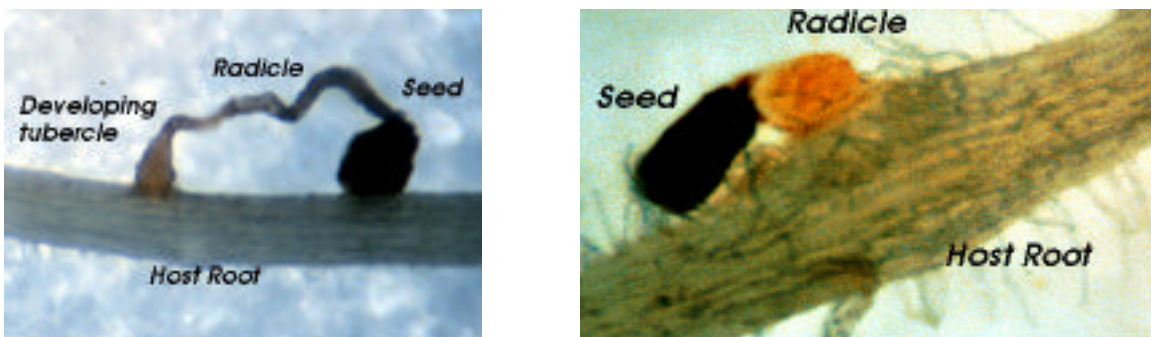


Figure 3.5. Early small broomrape (*O. minor*) attachments formed on red clover roots.

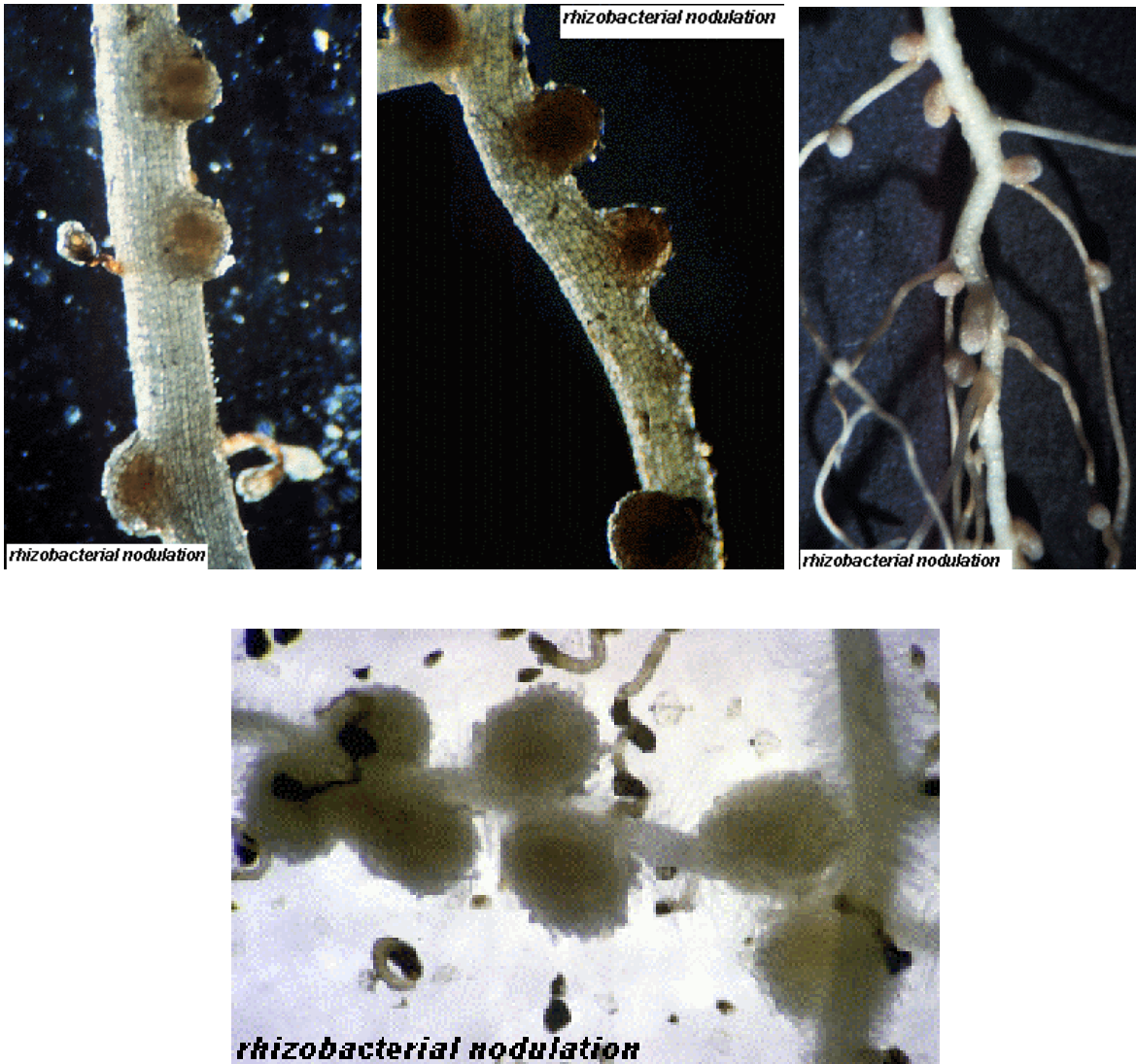


Figure 3.6. Nodules formed on red clover.

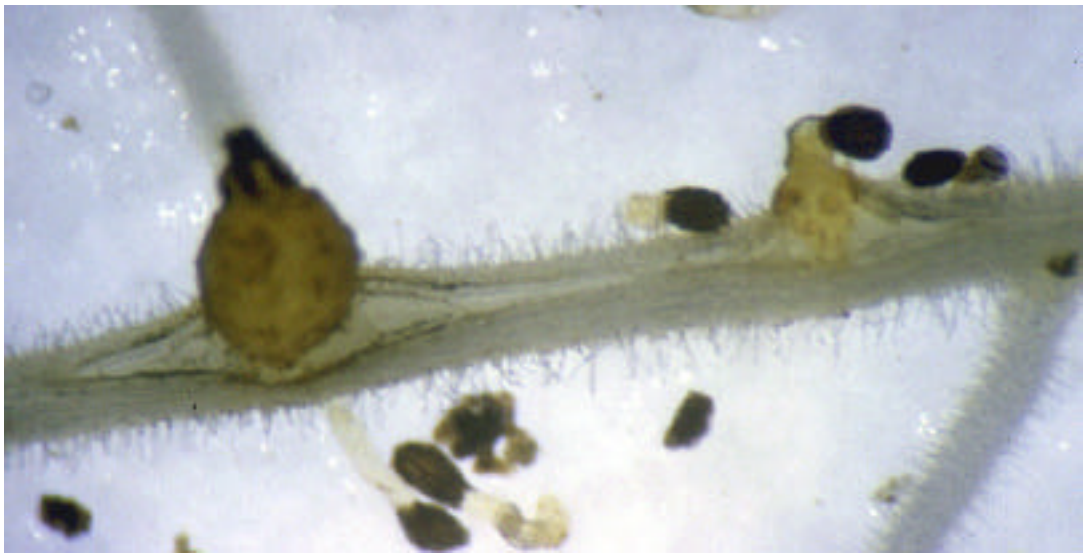


Figure 3.7. Root cortex splits induced by small broomrape (*O. minor*) attachments.

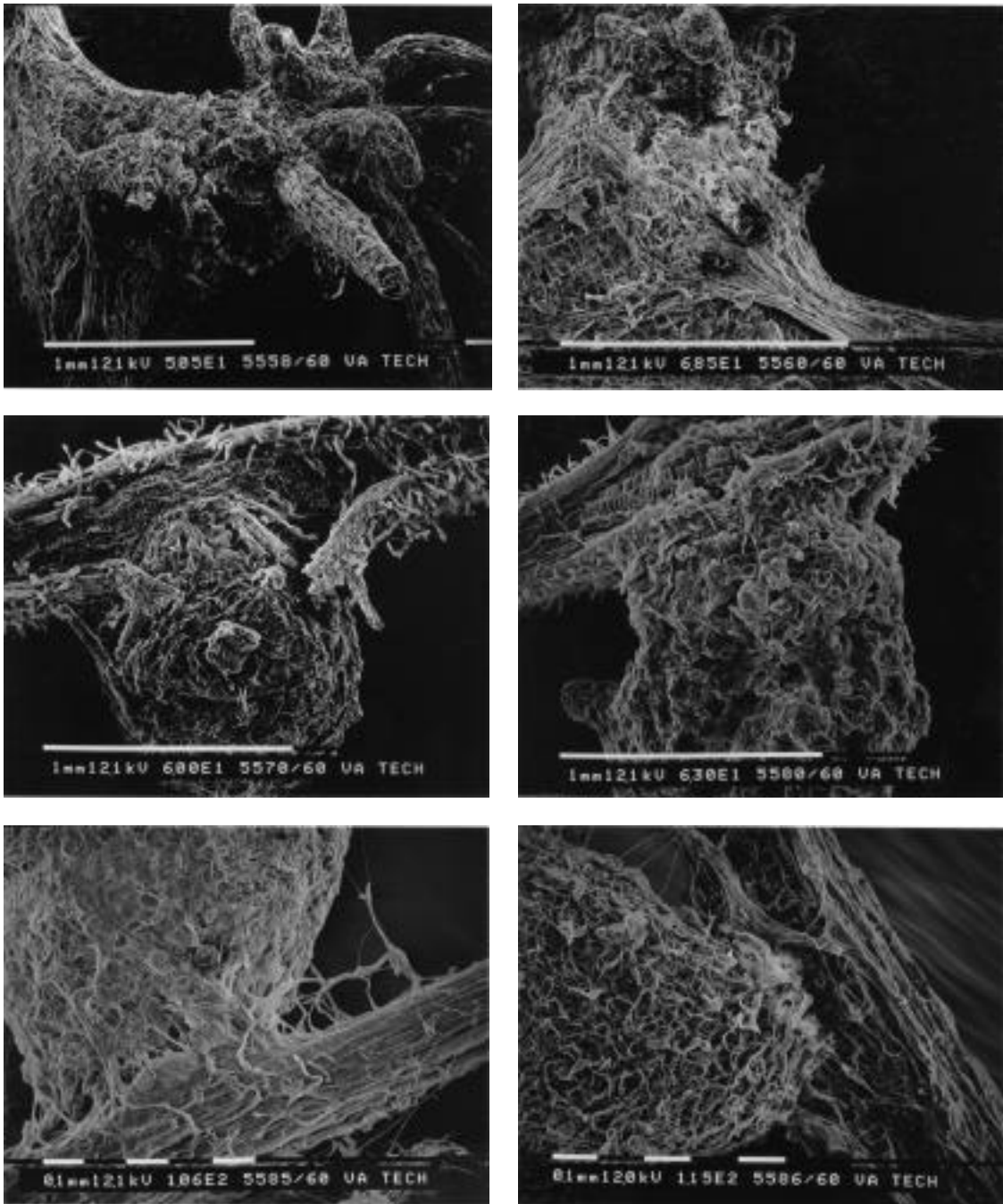


Figure 3.8. SEM review of nodules and small broomrape (*O. minor*) attachments. 5558, 5560, 5570, 5580 – *O. minor* attachments; 5585, 5586 – rhizobacterial nodules.

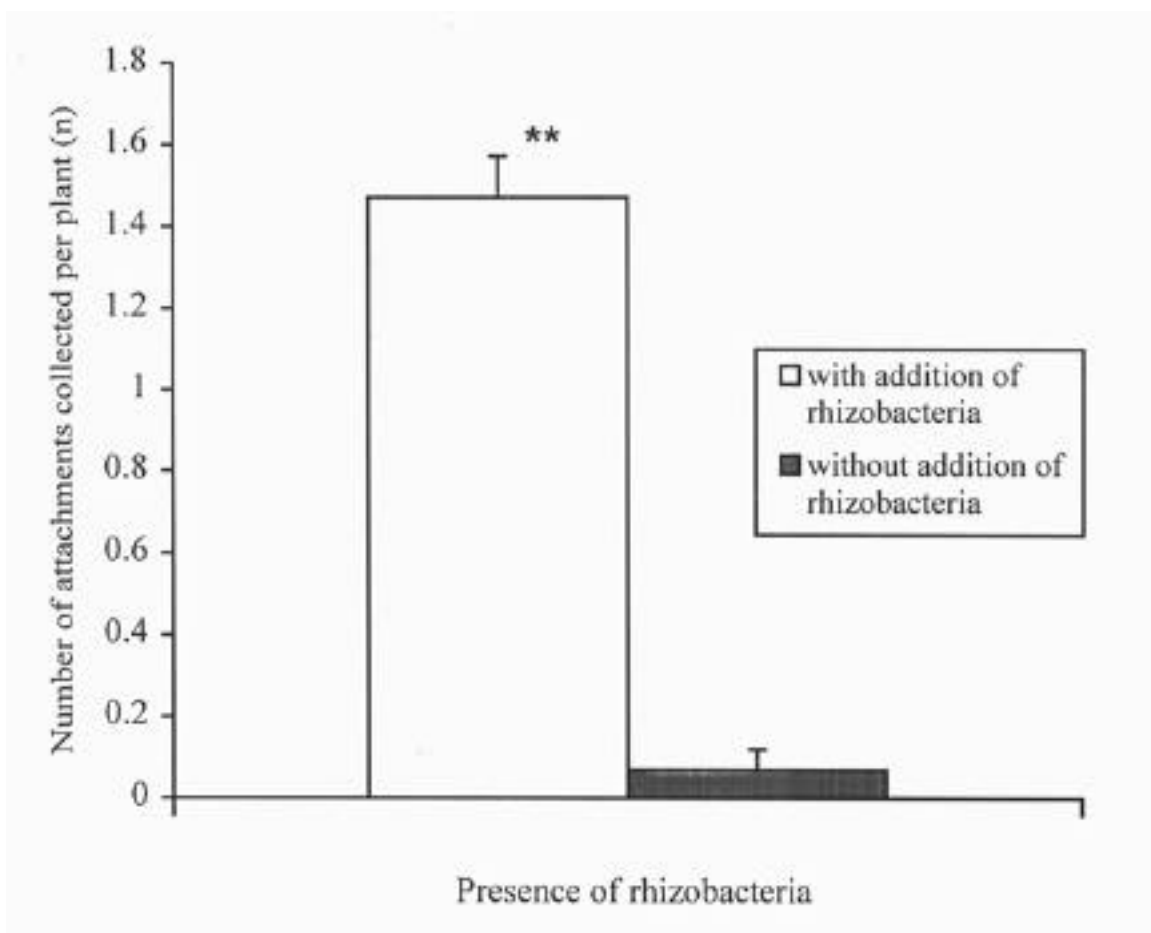


Figure 3.9. Variation in small broomrape (*O. minor*) number of attachments in aseptic tests with and without addition of rhizobacteria. Data are the means of *O. minor* attachments collected 45 DAT from 14 jars with and 14 jars without addition of the rhizobacteria. Each jar contained 10 replications of red clover plants. [** Denotes statistically significant difference ($\alpha = 0.01$), (SAS 1989)].

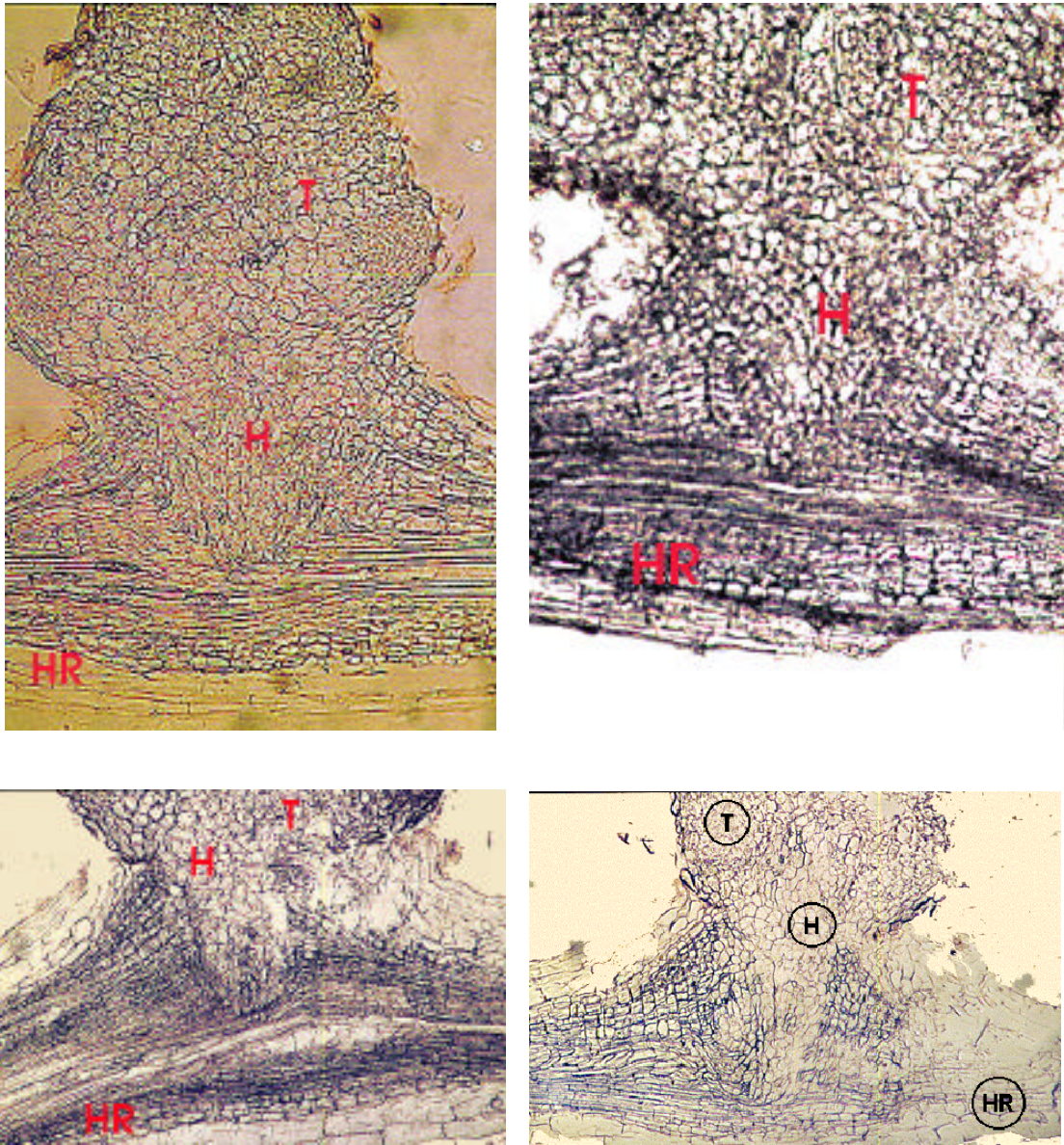


Figure 3.10. Longitudinal sections of small broomrape (*O. minor*) attachments formed on red clover roots. T – tubercle of the parasite; H – haustorium of the parasite; HR – host root.