Chapter 2. COMPARISON OF SMALL BROOMRAPE (*Orobanche minor* Sm.) AND EGYPTIAN BROOMRAPE (*Orobanche aegyptiaca* Pers.) PARASITIZATION OF RED CLOVER (*Trifolium pratense* L.).

2.1 INTRODUCTION

Small broomrape (*Orobanche minor* Sm.) and Egyptian broomrape (*Orobanche aegyptiaca* Pers.) are phanerogamic holoparasites that subsist on the roots of a variety of the leguminous and other broadleaf crops from which the parasites derive carbon, water, and nutrients needed for further growth. Both *O. minor* and *O. aegyptiaca* are devastating parasites of a number of economically important plants, including broad bean, also known interchangeably as faba bean, (*Vicia faba* L.), pea (*Pisum sativum* L.), carrot (*Daucus carota* L.), sunflower (*Helianthus annuus* L.), alfalfa (*Medicago sativa* L.), red clover (*Trifolium pratense* L.), white clover (*Trifolium repens* L.), and some varieties of vetch (*Vicia* spp.). Both *O. minor* and *O. aegyptiaca* lack chlorophyll, and hence lack the ability to synthesize their own food (Baccarini and Melandri 1967; Saghir et al. 1973). The growth of the parasitic plants occurs at the expense of water, minerals, and organic compounds obtained from the host plant, which consequently leads to host plant exhaustion and a lower biomass accumulation (Baker et al. 1995). In case of a severe infestation, parasitism could lead to considerable damage and retardation of the host growth, resulting in complete crop failure (Foy et al. 1989).

O. minor and *O. aegyptiaca* produce fine, almost dust-like seeds in large quantities (Kadry and Tewfic 1956). Broomrape seeds remain dormant in the period of after-ripening, and do not germinate until they are in the vicinity of a suitable host and the environmental conditions are favorable. Moreover, in order to germinate, *Orobanche* spp. seeds must meet two basic requirements (Foy et al. 1989). First, broomrape seeds require a period of preconditioning, which could last from a few days to several weeks, in a warm and moist surrounding (Jain and Foy 1987; Kasasian 1973). The second requirement is the presence of the germination stimulant, a chemical signal from host root exudates, that will 'trigger' the germination of preconditioned seeds. Upon germination, broomrape seeds develop tube-like radicle. If the germinating broomrape seed is within reach of a host root, the radicle can attach to the root surface. The natural germination stimulant(s) for *Orobanche* spp. has not been found, although, in vitro some broomrapes responded well to artificial plant growth stimulants such as strigol, and its analogs, GR-7, 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, inhibits the vascular system, and begins to draw 'food' from the host plant. The portion of the parasite that remains outside of the root tissue develops into a tubercle (Foy et al. 1989; Parker and Riches 1993). In turn, the tubercle produces a shoot that emerges above the soil surface and forms the 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; Musselman 1994; Sauerborn 1991). Often, *O. minor* attachments form a characteristic swelling around and at the point of penetration of the host root cortex. In faba bean similar swellings around *Orobanche crenata* Forsk. attachments were assumed to be bacterial nodules, which aided broomrape penetration of the host cortex (Petzoldt 1979).

Legumes, several of which are parasitized by *O. aegyptiaca* and *O. minor*, form a symbiotic relationship with the nitrogen-fixing rhizobacteria belonging to family Rhizobiaceae, providing bacteria with an ecological niche in the nodules, structures formed on the roots of the legume plant upon inoculation with rhizobacteria (Hirsch 1992; Vance et al. 1988). Rhizobia grow well in the presence of O_2, and utilize relatively simple carbohydrates and amino compounds. Most of the known strains have not been found to fix N in the free-living form except under special conditions. Most rhizobia produce white colonies when cultured in yeast-mannitol medium, with optimum growth at a temperature range of 25 to 30 C and a pH of 6.0 to 7.0.

Rhizobia are facultative microsymbionts that live as normal components of the soil microbial population when not living symbiotically in the root nodules of the host legume. Outside the root nodule, rhizobia are mostly found on the root surface (rhizoplane), and soil around and close to the root surface (rhizosphere). Numbers of rhizobia in the soil can range from undetectable to 1,000,000 rhizobia g^{-1} soil, and can be affected by the excretion of nutrients by plant roots, especially the host legume, moisture, temperature, soil pH, and salt content of the soil (Somasegaran and Hoben 1994).

Free-living rhizobia in the soil can enter the root hairs of the susceptible host legume through a series of complex interactions, which begin with a signal exchange, followed by the adhesion of rhizobia to the surface of the root hair (Franssen et al. 1992; Hirsch 1992). Adhesion is followed by deformation, and curling of the root hair, which results in the characteristic shepherd's crook appearance (Pawlowski and Bisseling 1996; Somasegaran and Hoben 1994). The hypha-like infection thread develops gradually in the root hair as a tubular structure that is actually an invagination of the root hair wall (Berry and Sunell 1990). Containing high numbers of rhizobial cells, the infection thread branches through the root cortex passing close to the host cell nuclei (Callaham and Torrey 1981; Somasegaran and Hoben 1994; van Spronsen et al. 1994).

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 invade host legume (Petzoldt 1979). Schmitt et al. (1979) concluded that *Orobanche* infestation does not occur before formation of legume nodules on the host plant. Moreover, infestations are more intense in aerobic conditions (Cubero 1973) when more nodules are produced. However, others have not confirmed these reports. In general, broomrape parasitism and rhizobacterial nodulation appear to follow a number of similar steps in their signal exchanges with the host. These steps include exudation by the roots of the host plant of a variety of stimulatory factors recognized by the invading parasite, similarly to rhizobacteria. Upon recognition of the host plant stimulants, broomrapes and bacteria release their own chemical signals, which help overcome the defense systems of the plant and gain penetration. Studies on the host-parasite interaction could help in gathering more information on parasitic weeds' biology and anatomy of the parasitism, which currently is limited. This study was conducted to investigate the possibility of a relationship between rhizobacterial nodulation and selected *Orobanche* spp. parasitization of red clover.

2.2 MATERIALS AND METHODS

2.2.1 PLANT MATERIAL

Dr. Y. Kleifeld[1] provided seeds of Egyptian broomrape (*O. aegyptiaca* Pers.). Seeds of small broomrape (*O. minor* Sm.) were provided by Dr. R. Eplee[2], from the stock collected in Thomas Co., GA, USA. *Orobanche* 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 Co[3]. Red clover seeds were refrigerated at 4 C in plastic bag until used. Inoculum culture, *Rhizobium leguminosarum* bv. *trifolii*, was purchased from the American Type Culture Collection[4] and cold-stored at 4 C until use. According to USDA APHIS[5], *Orobanche* spp. are considered to be noxious weeds in the United States; thus, handling of the broomrape material was done in strict quarantine.

2.2.1.1 Orobanche spp. Seed Preparation

O. minor and *O. aegyptiaca* seeds were surface-disinfected for 15 min in a mixture of 1%

sodium hypochlorite (commercial bleach) and 1% v/v Tween 20â [6] following a 15-s immersion in 70% aqueous ethyl alcohol[7] (by vol). Subsequently, seeds were rinsed in autoclaved[8] distilled water three times, 15 min each time. *Orobanche* spp. seeds were placed in 9-cm-diam Petri dishes[9] between two glass-fiber filter paper (GFFP)[10] disks. Sealed with parafilm[11], Petri dishes were stored at 25 C in the dark room.

2.2.1.2 Red Clover Seed Preparation, Inoculation, and Planting

Red clover seeds were surface-disinfected, as described for broomrape seeds. A batch of surface-disinfected red clover seeds was planted in plastic trays[12] (Figure 2.1.) in autoclaved vermiculite[13], which was used as a soil substitute. A second batch (10 g) was inoculated with rhizobium-containing peat powder[14], prior to planting 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 K_2HPO_4[15] (0.5 g L^{-1}), $MgSO_4$ x $7H_2O$[16] (0.2 g L^{-1}), NaCl[17] (0.1g L^{-1}), dissolved in distilled (autoclaved) water (1.0 L), which was followed by the addition of the D-mannitol[18] (10 g L^{-1}) and yeast extract[19] (0.5 g L^{-1}). While the mixture was continuously agitated, the pH of the liquid was adjusted to 6.8 with 0.1 N 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 inoculant[20]. A batch of 80 g of fresh inoculant (21.5% moisture content) was dried in an oven[21] for 36 h. Two autoclaved jars[22] 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 to 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% sucrose[23] solution) and the semi-powdered inoculant (0.5 g peat powder; 7 x 10^4 rhizobia cells/g of inoculated red clover seed)[24] 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. Excess sticker will make the seeds clump together and too little will cause uneven coating with the inoculant; therefore, the size of the seeds and the experience of the applicator determine the amounts of the ingredients in this two-step method (Somasegaran and Hoben 1994).

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 a controlled

environment room under fluorescent lights. Light intensity was 100 µmol m^{-2} 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 solution[25] (Table 2.1.; 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; Table 2.1.), in order to stimulate nodulation.

2.2.2 POLYETHYLENE (PE) BAG EXPERIMENTS

Healthy, 20-d-old, red clover plants were transplanted into polyethylene (PE) plastic bags[26]. Preconditioning and germination of *Orobanche* spp. took place in the bags. Red clover plants inoculated with *Rhizobium leguminosarum* bv. *trifolii* were grown separately from non-inoculated plants. Small broomrape and Egyptian broomrape were grown on red clover for 42 d in the PE bags (15 x 30 cm), each containing a glass fiber (GF/A)[27] 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, *Orobanche* spp. seeds (0.015 g, by weight from stock collection) were uniformly dispersed in an 80-cm^2 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 (Figure 2.2.). Boxes were placed in a 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), which were injected into the bags through a small opening cut on the side of each bag.

The experiments followed a random design with 30 replications of PE bags each containig two red clover plants. Data on broomrape percent germination and number of attachments to the red clover were collected every 3 d for a period of 42 d. All data were subjected to analysis of variance, using SAS statistical software (SAS 1989).

2.2.2.1 Examining Nodules and Broomrape Attachments

Broomrape development was periodically observed under the stereoscopic microscope[28] to determine the rate of broomrape seed germination and the number of parasitic attachments on the host. Data on percent germination were collected from randomly selected 1-cm^2 areas along the red clover roots (four areas per bag). Red clover root system was examined to determine the number of nodules formed. Thin slices of the nodules, cut with a razor blade and floated in a drop of water on a microscope slide, were covered with a cover glass and examined under the microscope.

2.2.2.2 Isolation of Rhizobacteria

Ten nodules were collected from five randomly selected plants (two nodules from each). Nodules were severed from the roots by cutting the root about 0.5 cm on each side of the nodule. Nodules were removed using forceps on the root appendages to reduce the risk of damaging the nodule. Collected nodules were immersed in 95% ethanol followed by 5-min soaking in 2.5% (by vol) aqueous solution of sodium hypochlorite, according to Somasegaran and Hoben (1994). Surface-cleaned nodules were rinsed in autoclaved distilled water (five times, 10 min each). With a pair of blunt-tipped forceps nodules were crushed in a large drop of sterile water in a Petri dish, creating a "nodule suspension". One loopful of nodule suspension was streaked on a yeast-mannitol agar (YMA) plate. YMA plates were prepared by solidifying yeast-mannitol broth with 0.15% (by weight) agar[29]. Streaked with nodule suspension, plates were incubated in the dark at 25 C for 5 to 10 d. Plates were subjected to periodic observations of developing colonies. Similar isolations were made from the rhizobium-containing peat powder and yeast-mannitol broth. Colonies obtained from the three different sources were compared, with yeast-mannitol broth colonies accepted as the standard.

2.3 RESULTS AND DISCUSSION

2.3.1 POLYETHYLENE (PE) BAG EXPERIMENTS

Addition of the rhizobacteria influenced *O. minor* development, as percent germination

of the parasite and number of attachments per host plant were both significantly higher in the treatments inoculated with *Rhizobium leguminosarum* bv. *trifolii.* First germinating seeds were observed 6 days after transplanting (DAT) (Figure 2.3.), and first attachments were observed at 15 DAT (Figure 2.4.). A statistically significant difference ($\alpha = 0.05$) was not observed in percent germination of *O. minor* seeds until 27 DAT, and 30 DAT in average number of attachments per host plant. Perhaps it is a reflection of the placement of the broomrape seeds in the PE bag profile. Root systems of red clovers needed time to develop and reach the zone of *O. minor* seed location.

In all experiments *O. minor* attachments were accompanied by a formation of a characteristic swelling of the root tissue around the point of parasite penetration (Figure 2.5.) which was not present at the initial stages (Figure 2.6.). Similar swellings of the root tissue around broomrape attachment were reported earlier by Petzoldt (1979) in studies of *O. crenata* parasitism of faba bean.

O. minor attachments did not appear to be situated over the distinct and developed nodules (Figure 2.7.). Not only did the swelling beneath the tubercle of the parasite had a different morphological appearance, but also it often induced splits of the root cortex (Figure 2.8.) uncharacteristic to nodulation. Microscopic observations indicated that the enlargement of the host root tissue could be the result of parasite protrusion, root cortex enlargement, or both. Nevertheless, the appearance of the parasitic connections does not preclude the possibility of rhizobacteria involvement in the host-parasite relationships. Perhaps parasite could utilize the signal exchange between rhizobia and the host plant for its advantage to gain penetration.

O. aegyptiaca treatments served as another example of broomrape parasitism in response to the inoculation of the host. Observations of *O. aegyptiaca* percent germination and number of attachments provided information for comparative analysis in the study of *Orobanche* spp. parasitism of red clover. First germinated seeds of *O. aegyptiaca* were observed 6 DAT plants into PE bags, in both inoculated and non-inoculated treatments (Figure 2.9.). First *O. aegyptiaca* attachments on non-inoculated red clovers were observed at 15 DAT, and at 27 DAT on inoculated plants (Figure 2.10.). *O. aegyptiaca* germination and number of attachments did not appear to have any preference for the presence of rhizobacteria, as no significant difference ($\alpha = 0.05$) in percent seed germination and number of parasitic attachments between inoculated and non-inoculated plants was observed. *O. aegyptiaca* attachments appeared to have preference for the healthiest host irrespective of inoculation, and the number of attachments did not differ significantly between the inoculated the non-inoculated treatment. It appears that *O. aegyptiaca* depends on the well-being of the host rather than on inoculation.

Perhaps *Orobanche* spp. penetration is coordinated with the host. In case of *O. aegyptiaca* germination, penetration, and attachment, the presence of rhizobacteria did not

appear to play a major role, and most of the attachments were observed in the bags with non-inoculated red clover plants. However, non-inoculated red clover plants appeared to be healthier than inoculated red clover, and the low number of attachments of the parasite did not seem to play a major role in the well-being of the host. Perhaps inoculated plants grown in the artificial environment and receiving N-deficient half-strength Hoagland's solution were at a disadvantage in obtaining necessary nutrients for normal growth. Inoculated plants had slightly more chlorotic leaves and partially retarded growth in comparison to non-inoculated plants, which received normal half-strength Hoagland's solution. A low number of attachments in *O. aegyptiaca* treatments may reflect the specificity of Egyptian broomrape-red clover interaction. Although, *O. aegyptiaca* is not considered as a common red clover parasite, the parasite recognized the chemical stimulant(s) in red clover root exudates, and was able to establish itself on the host roots. However, whether *O. aegyptiaca* and *O. minor* seeds germinate in response to the same germination stimulants in red clover root exudates remains unknown. In addition, inoculation of red clover could have also change the quantity as well as the structure of the germination stimulants; however, *O. aegyptiaca* percent germination and number of attachments in inoculated treatments did not differ significantly from those in non-inoculated treatments. Moreover, *O. aegyptiaca* did not induce the formation of the swelling at the point of attachment (Figure 2.11.), typical among *O. minor* attachments, although the diameter of the root increased due to the parasite protrusion. Detailed anatomical analysis of the infected zones on the roots of the host revealed the location of the haustorial cells of *O. aegyptiaca*. Intrusive cells, which spearheaded the penetration of the parasite appeared to ramify through the host plant tissues by elongation without dividing. There were no signs of host cells proliferation in any of the observed attachments. While some of the host cells around *O. aegyptiaca* penetrating cells appeared to be crushed it was impossible to determine whether this was due to enzymatic or mechanical forces induced by the parasite or was an artifact of the slide preparation.

2.3.2 EXAMINING NODULES AND PARASITIC ATTACHMENTS

Due to the presence of *leghemoglobin*, active N-fixing nodules had a characteristic pink, reddish, or brownish color (Figure 2.12.). Senescent nodules were usually grayish green. When active nodules were exposed to the sunlight they developed a greenish exterior, due to chlorophyll development on the cortical region of the nodule (Somasegaran and Hoben 1994). There was no spontaneous nodulation observed in the non-inoculated treatments. Moreover, the average number of nodules formed per plant in the inoculated treatments was similar between the *Orobanche* spp. (Figure 2.13), indicating that the presence of one of the either *Orobanche* spp.

did not differ in affecting the number of nodules formed.

Exteriors of *O. minor* live tubercles were of a brown to rust color (Figure 2.14.) and brighter than the color of the nodules. Attachments of *O. aegyptiaca* were yellow to pale-green.

Crushed nodules collected from the treatments produced rhizobacterial colonies similar to those obtained from the yeast-mannitol broth (YMB) and rhizobium-containing peat powder. *Orobanche* spp. attachments taken from the plants inoculated with rhizobia also gave rise to colonies similar to those in YMB. On the contrary, attachments collected from the non-inoculated plants did not produce identifiable rhizobaterial colonies, even when plates were left for a period of 4 wk. However, the plates streaked with the samples from the non-inoculated plants produced some fungal growth, which was expected since the plant materials were grown in the septic environment.

2.4 CONCLUSIONS

Experiments were conducted to evaluate the role of *Rhizobium* nodulation in facilitation of *O. minor* and *O. aegyptiaca* parasitization of red clover. Host plants inoculated with *Rhizobium leguminosarum* bv. *trifolii* showed an increase in stimulation of *O. minor* seed germination and had a higher number of parasitic attachments than non-inoculated plants.

O. aegyptiaca parasitization was found to be not dependent on the presence of the rhizobacteria. On the contrary, *O. minor* germination was significantly increased by inoculating hosts with rhizobia, and the number of parasitic attachments, which were accompanied by swelling of host root tissue at the point of parasite penetration. Although we did not find attachments situated over distinct and mature nodules, addition of rhizobia could play a role other than serving as a point of parasite entry, such as influencing the host-parasite signal exchange. Stimulatory effects of rhizobacterial nodulation on *O. minor* parasitism support previous reports of increases in broomrape infestations in the presence of nodule-forming bacteria (Petzoldt 1979; Schmitt et al. 1979). However, we find that *O. minor* appears to follow a different pathway than that proposed for *O. crenata* on faba bean where the parasite was assumed to penetrate the host indirectly over bacterial nodules (Petzoldt 1979). Parasitization "over-the-nodule" would seem likely to occur at the point of nodule connection to the host plant, helping the parasite to avoid traversing bacterial nodule tissue in order to reach the vascular system of the host. Another possibility is the parasitization of the nodules and gathering of the nutrients and minerals from the nodules rather than the host. Although no direct parasitization of the mature nodules was observed for either *O. minor* or *O. aegyptiaca*, the possibility of over-the-nodule penetration remains. In vivo verification of the results of this study is necessary.

However, a conclusion can be made that inoculation could to be a valuable tool in the use of trap or catch crops, or help in the isolation and identification of the natural growth stimulants. Recent discoveries of the enzymes that degrade host cell wall components in *O. aegyptiaca* exudates (Shomer-Ilan 1994), suggests a close and complex signal exchange between the host plant and the parasite, in which the latter must suppress or work in coordination with the host's defense mechanism(s) in order to gain penetration.

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.

2.5 SOURCES OF MATERIALS

[1] Dr. Y. Kleifeld, Newe Ya'ar Research Center, Ramat Ishay 30095, Israel.

[2] Dr. R. Eplee, Oxford Plant Protection Center, USDA-APHIS-PPQ, 901 Hillsboro St., Oxford, NC 27565.

[3] Red clover seeds, commercial mix. Blacksburg Feed and Seed Co., 1212 N Main St., Blacksburg, VA 24060.

[4] Inoculum culture. *Rhizobium leguminosarum* bv. *trifolii*, cat. # 14479; American Type Culture Collection ®, 12301 Parklawn Drive, Rockville, MD 20852.

[5] USDA APHIS. Executive Branch of the US Government: United States Department of Agriculture: Animal and Plant Health Inspection Service.

[6] Tween 20 ®, polyoxyethylene sorbitan monolaurate, a surfactant. ICN Biomedicals, Inc., 1263 South Chillicote Road, Aurora, OH 44202.

[7] Ethyl alcohol, 200 proof. Aaper Alcohol and Chemical Co., Shelbyville, KY 40065.

[8] Thermatic 60 autoclave. Castle Sybron, 1777 E. Henrietta Rd., Rochester, NY 14602.

[9] Plastic Petri dish (08-757-12). Fischer Scientific Co., 711 Forbes Ave., Pittsburgh, PA 15219.

[10] Glass-fiber filter paper (GFFP). Fischer Scientific Co., 711 Forbes Ave., Pittsburgh, PA 15219.

[11] Parafilm M, laboratory film. American National Can, Greenwich, CT 06830.

[12] Plastic trays. 2 qt rectangle Rubbermaid â food storage containers; cat. 42733WX; Consolidated Plastics Co. Inc., 8181 Darrow Road, Twinsburg, OH 44087.

[13] Vermiculite. Wetsel Seed Co., PO Box 791, Harrisonburg, VA 22801.

[14] Rhizobium-containing peat powder. Prepared according to Somasegaran and Hoben (1994) using yeast-mannitol broth (YMB).

[15] Potassium phosphate dibasic powder (3254). J. T. baker Chemical Co., Phillipsburg, NJ.

[16] Magnesium sulfate crystal (M-63). Fischer Scientific Co., 711 Forbes Ave., Pittsburgh, PA 15219.

[17] Sodium chloride (S-9625). Sigma Chemical Co., PO Box 14508, St. Louis, MO 63178.

[18] D-mannitol (Mannite, M-1902). Sigma Chemical Co., PO Box 14508, St. Louis, MO 63178.

[19] Yeast extract (Y-0500). Sigma Chemical Co., PO Box 14508, St. Louis, MO 63178.

[20] Alfalfa-clover inoculant (nitrogen fixing bacteria) peat powder (5 oz. pack). Southern States Cooperative, Richmond, VA 23230.

[21] Stabil-Therm (OV-500C; laboratory oven). Blue M Electric Co., Blue Island, IL 60406.

[22] Vessels for plant tisue culture (baby food jars) (V8630; 100 ml). Sigma Chemical Co., PO Box 14508, St. Louis, MO 63178.

[23] Sucrose. Sigma Chemical Co., PO Box 14508, St. Louis, MO 63178.

[24] Rhizobia counts in the peat powder are approximate values, based on inoculant of 1.4×10^7 rhizobacterial cells / g of peat powder depositing approximatelly 87.5 cells / inoculated red clover seed.

[25] Half-strength Hoagland's nutrient solution. Prepared in the laboratory according to the Hoagland and Arnon (1950).

[26] Polyethylene (PE) plastic bags. Fischer Scientific Co., 711 Forbes Ave., Pittsburgh, PA 15219.

[27] Glass-fiber filter paper (GF/A). Whatman International, Maidstone, UK, ME16 0LS.

[28] Leica GZ7 Stereomicroscope. Leica Inc., PO Box 123, Buffalo, NY 14240-0123.

[29] Agar (A-1296). Sigma Chemical Co., PO Box 14508, St. Lois, MO 63178.

2.6 LITERATURE CITED

Abdel Halim, M. A., M. A. Amar, A. Hazem, and S. Abdel Hafeez. 1975. Contributions to the germination of *Orobanche* seeds with reference to root exudates of host. Ann. Agric. Sci. 20: 127-139.

Baccarini, A. and B. A. Melandri. 1967. Studies on *Orobanche hederae* physiology: Pigments and CO_2 fixation. Physiol. Plant. 20:245-250.

Baker, E. R., J. D. Scholes, M. C. Press, and W. P. Quick. 1995. Effects of the holoparasite *Orobanche aegyptiaca* on the growth and photosynthesis of its tomato host. Aspects Appl. Biol. 42: 141-148.

Berry, A. M. and L. A. Sunell. 1990. The infection process in nodule development. *In*: C. R. Schwintzer and J. D. Tjepkema, eds. The Biology of *Frankia* and *Actinorhizal* Plants. pp. 61-81.

Callaham, D. A. and J. G. Torrey. 1981. The structural basis of infection of root hairs of *Trifolium repens* by *Rhizobium*. Can. J. Bot. 59: 1647-1664.

Cubero, J. I. 1973. Resistance to *O. crenata* in *Vicia faba*. Proc. Eur. Weed Res. Counc. Symp. Parasitic Weeds. pp. 205-217.

Foy, C. L., R. Jain, and R. Jacobsohn. 1989. Recent approaches for chemical control of broomrape (*Orobanche* spp.). Rev. Weed Sci. 4:123-152.

Franssen, H. J., I. Vijn, W. C. Yang, and T. Bisseling. 1992. Developmental aspects of the *Rhizobium*-legume symbiosis. Plant Mol. Biol. 19: 89-107.

Hirsch, A. M. 1992. Developmental biology of legume nodulation. New Phytol. 122: 211-237.

Hoagland, D. R. and D. I. Arnon. 1950. The Water Culture Method for Growing Plants Without Soil. California Agricultural Experiment Station Circ. 347. Berkeley, CA: University of California. 32 p.

Jain, R. and C. L. Foy. 1987. Influence of various nutrients and growth regulators on germination and parasitism of *Orobanche aegyptiaca*. *In*: H. Chr. Weber and W. Forstreuter, eds. Proc. 4th Int. Symp. Parasitic Flowering Plants, Marburg, FR Germany, Philipps-Universität. pp. 427-436.

Johnson, A. W., G. Roseberry, and C. Parker. 1976. A novel approach to *Striga* and *Orobanche* control using synthetic germination stimulants. Weed Res. 16: 223-227.

Kadry, A.E.El.R. and H. Tewfic. 1956. Seed germination in *Orobanche crenata*. Sven. Bot. Tidskr. 50: 270-286.

Kasasian, L. 1973. Miscellaneous observations on the biology of *Orobanche crenata and O. aegyptiaca*. Proc. Eur. Weed Res. Soc., Symp. Parasitic Weeds, Malta, Wageningen, The Netherlands. pp. 68-75.

Littell, R. C., G. A. Milliken, W. W. Stroup, and R. D. Wolfinger. 1996. SASâ System for Mixed Models. SAS Institute, Cary, NC. 633 p.

Musselman L. J. 1994. Taxonomy and spread of *Orobanche*. *In*: A. H. Pieterse, J.A.C. Verkleij and S. J. ter Borg, eds. Biology and management of *Orobanche*, Proc. of the 3rd Int. Workshop on *Orobanche* and related *Striga* research. Amsterdam, The Netherlands, Royal Tropical Institute. pp. 27-35.

Parker, C. and N. Dixon. 1983. The use of polyethylene bags in the culture and study of *Striga* spp. and other organisms on crop roots. Ann. Appl. Biol. 103: 485-488.

Parker, C. and C. R. Riches. 1993. Parasitic Weeds of the World: Biology and Control. Wallingford, UK: CAB International. 332 p.

Pawlowski, K. and T. Bisseling. 1996. Rhizobial and Actinorhizal symbioses: what are shared features? Plant Cell. 8: 1899-1913.

Petzoldt, K. 1979. Bacterial nodules of *Rhizobium leguminosarium* and *Orobanche crenata* germination and penetration on broadbeans with an integrated control program. Proc. 2nd Int. Symp. on Parasitic Weeds, USA. pp. 260-268.

Saghir, A. R. 1986. Dormancy and germination of Orobanche seeds in relation to control methods. *In*: S. J. ter Borg, ed. Proc. Workshop on Biology and Control of *Orobanche*. LH/VPO, Wageningen, The Netherlands. pp. 25-34.

Saghir, A. R., C. L. Foy, and K. M. Hameed. 1973. Herbicide effects on parasitism of tomato by hemp broomrape. Weed Sci. 21:253-258.

Sauerborn, J. 1991. Parasitic Flowering Plants: Ecology and Management. Verlag Joseph Margraf. Weikersheim, FR Germany. 129 p.

[SAS] Statistical Analysis Systems. 1989. The SAS System for Windows. Version 6. 12. Cary, NC: Statistical Analysis Systems Institute.

Schmitt U., K. Schluter, and P. A. Boorsma. 1979. Chemical control of *Orobanche crenata* in broad beans. FAO Plant Prot. Bull. 27 (3): 88-91.

Shomer-Ilan, A. 1994. Enzymes that degrade cell wall components are excreted by the haustorium of *Orobanche aegyptiaca* Pers. *In*: A. H. Pieterse, J.A.C. Verkleij, and S. J. ter Borg, eds. Biology and management of *Orobanche*. Proc. 3rd Int. Workshop on *Orobanche* and related *Striga* research. Amsterdam, The Netherlands, Royal Tropical Institute. pp. 255-260.

Somasegaran, P. and H. J. Hoben. 1994. Handbook for Rhizobia: Methods in Legume-*Rhizobium* Technology. New York: Springer-Verlag. 450 p.

Stewart, G. R., and M. C. Press. 1990. The physiology and biochemistry of parasitic angiosperms. Annu. Rev. Plant Physiol. Plant Mol. Biol. 41:127-151.

Vance, C. P., M. A. Egli, S. M. Griffith, and S. S. Miller. 1988. Plant regulated aspects of nodulation and N_2 fixation. Plant Cell Environ. 11: 413-427.

van Spronsen, P. C., A.A.N. van Brussel, and J. W. Kijn. 1994. Nod factors produced by *Rhizobium leguminosarum* biovar *viciae* induce ethylene-related changes in root cortical cells of *Vicia sativa* spp. *nigra*. Eur. J. Biol. 68: 463-469.

Zahran, M. A. 1982. Control of parasitic plants (broomrape and dodder) in different crops in Egypt. Final Technical Report, Agricultural Res. Program, PL 480. 53 p.

Table 2.1. Bacteria growth media and plant nutrient solutions.

	Solutions		
Constituents	YMB + A [a]	Strength Hoagland's [b]	Strength Hoagland's (-N) [c]
Agar	15% by vol.		
0.05M $Ca(H_2PO_4)_2$			3.15 g 0.250 L^{-1}
1M $Ca(NO_3)_2$		59 g L^{-1}	
0.01M $CaSO_4$			1.72 g L^{-1}
$CuSO_4 \cdot 5H_2O$		0.02 g L^{-1}	0.02 g L^{-1}
D-Mannitol	10 g L^{-1}	34 g L^{-1}	
H_3BO_3		0.715 g L^{-1}	0.715 g L^{-1}
Iron chelate DD		2.5 g L^{-1}	2.5 g L^{-1}
1M KNO_3		25.27 g 0.5 L^{-1}	
1M K_2HPO_4	0.5 g L^{-1}		
0.5M K_2SO_4			21.78 g 0.250 L^{-1}
1M $MgSO_4$ x $7H_2O$	0.2 g L^{-1}	61.62 g L^{-1}	61.62 g L^{-1}
$MnCl_2 \cdot 4H_2O$		0.45 g L^{-1}	0.45 g L^{-1}
NaCl	0.1 g L^{-1}		
$Na_2MoO_4 \cdot 2H_2O$		0.017 g L^{-1}	0.017 g L^{-1}
$ZnSO_4 \cdot 7H_2O$		0.06 g L^{-1}	0.06 g L^{-1}
Yeast extract	0.5 g L^{-1}		
Distilled water	1.0 L	1.0 L	1.0 L

[a] YMB + A – denotes yeast-mannitol broth with addition of agar, a complex used for the bacteria growth medium.

[b] Hoagland's – denotes half-strength Hoagland's solution.

[c] Hoagland's (- N) – denotes Nitrogen-deficient half-strength Hoagland's solution.

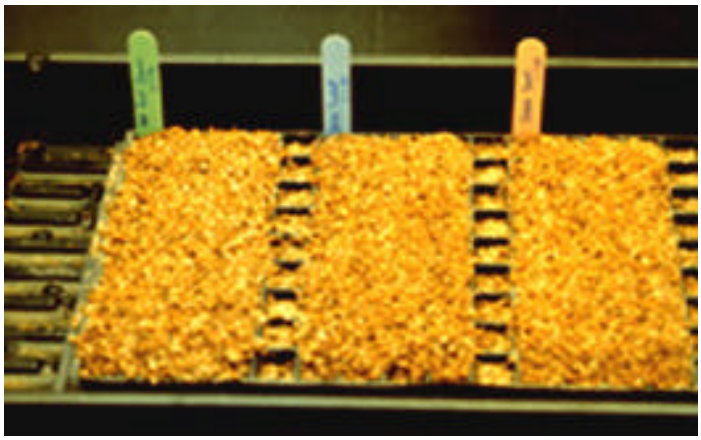

Figure 2.1. Plastic trays with seeded red clover.

Figure 2.2. Cardboard box with polyethylene bags (PE) containing red clover plants.

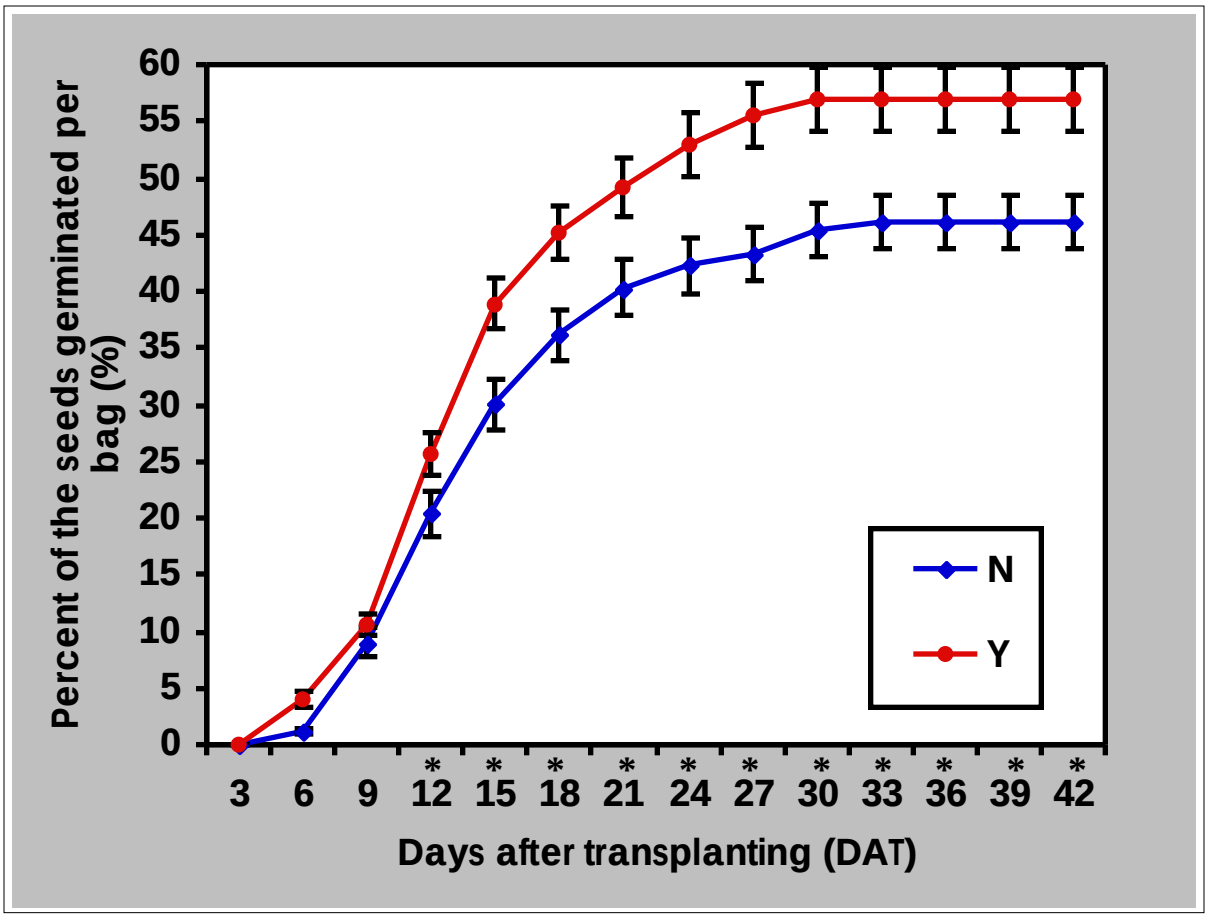

Figure 2.3. Small broomrape (*O. minor*) percent germination. Observations were made every 3 d for a period of 42 d. N – denotes non-inoculated treatments (without addition of rhizobacteria); Y – denotes inoculated treatments (with addition of rhizobacteria). Data are the means of 30 replications (α = 0.05; SASâ 1989). Asteric (*) – marks days when statistically significant difference between inoculated and non-inoculated treatments was observed.

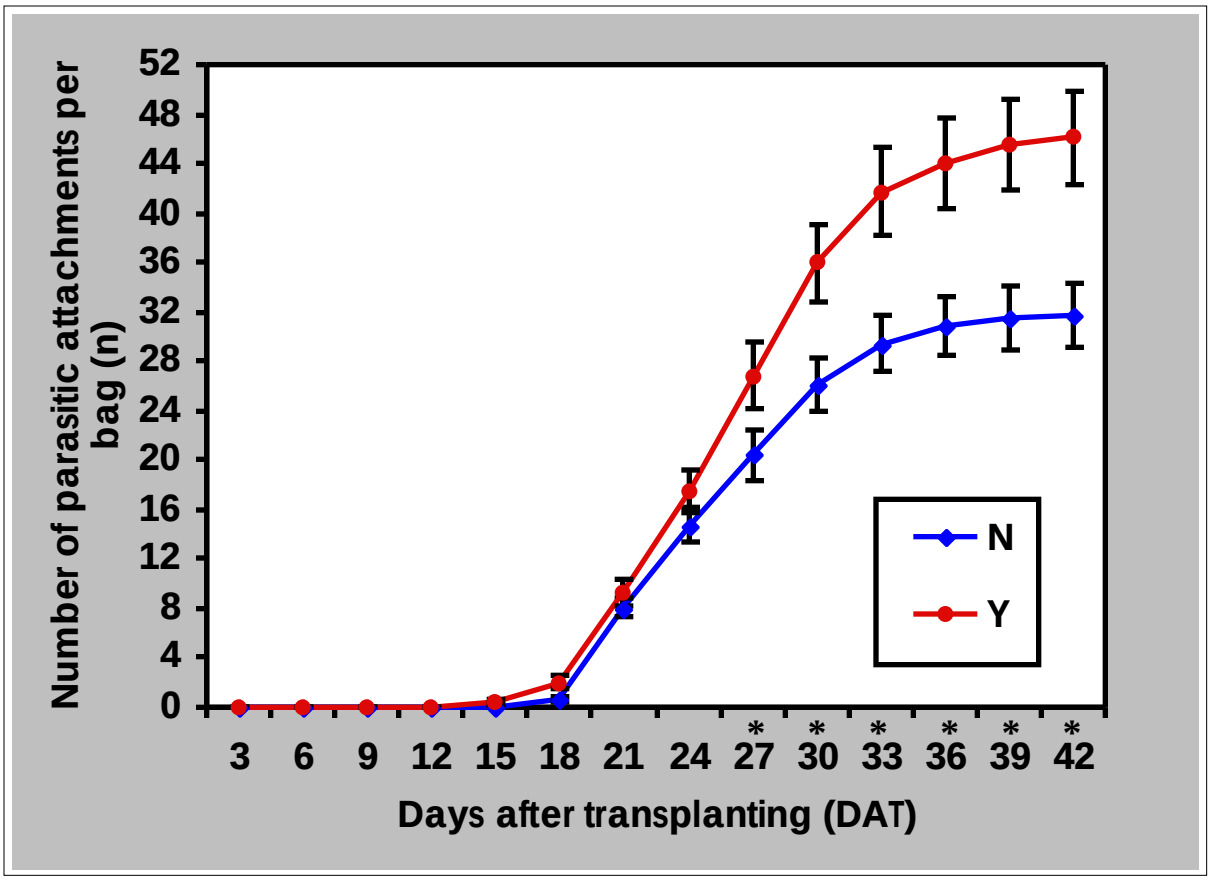

Figure 2.4. Number of small broomrape (*O. minor*) attachments formed on red clover roots. Observations were made every 3 d for a period of 42 d. N – denotes non-inoculated treatments (without addition of rhizobacteria); Y – denotes inoculated treatments (with addition of rhizobacteria). Data are the means of 30 replications (α = 0.05; SASâ 1989). Asteric (*) – marks days when statistically significant difference between inoculated and non-inoculated treatments was observed.

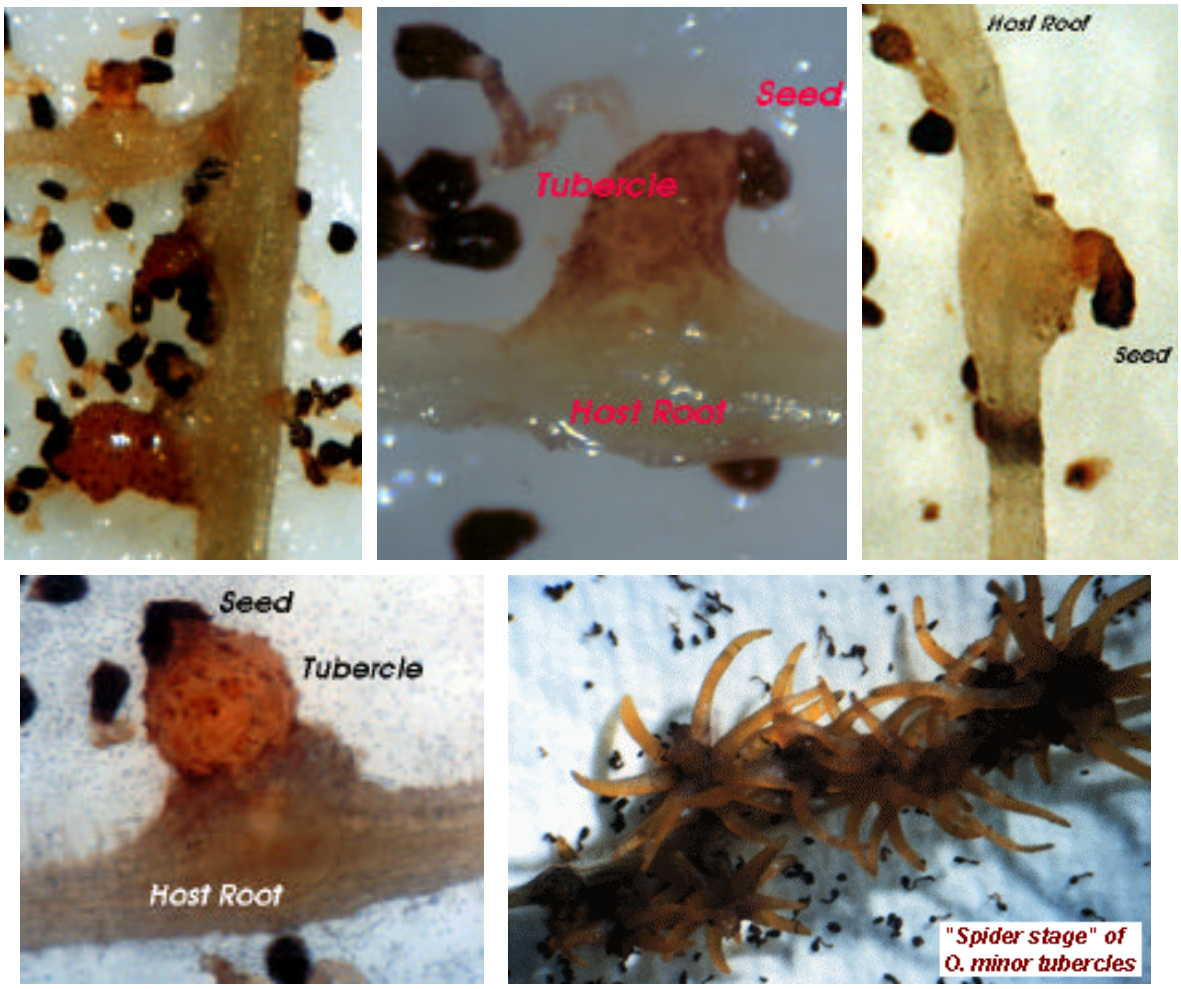

Figure 2.5. Small broomrape (*O. minor*) attachments formed on red clover roots.

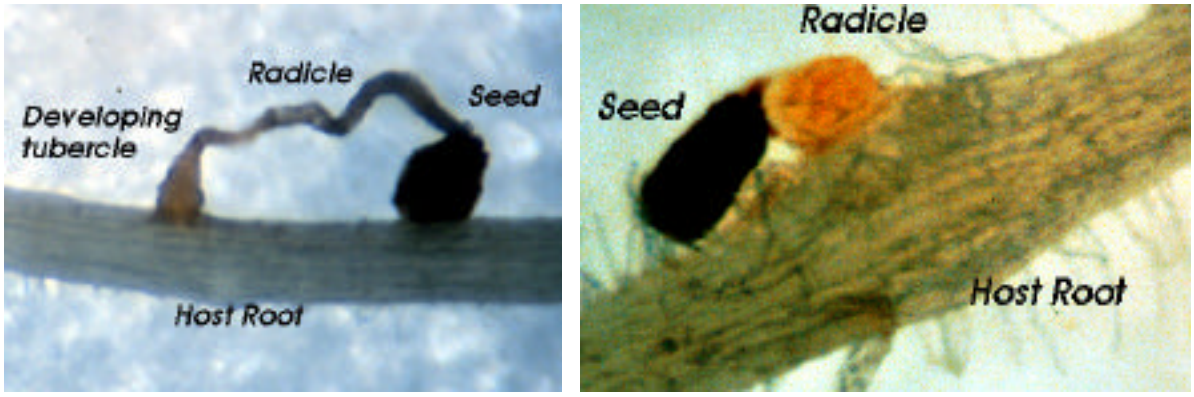

Figure 2.6. Early small broomrape (*O. minor*) attachments formed on red clover roots.

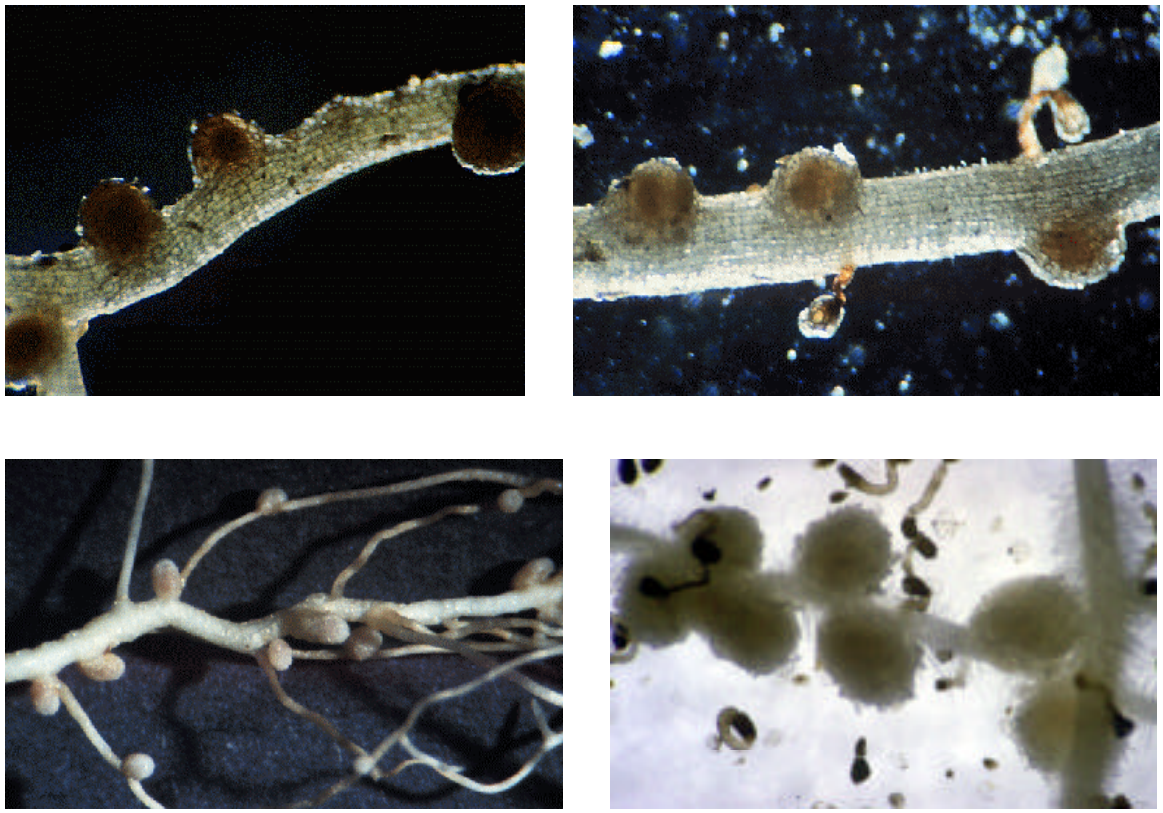

Figure 2.7. Nodules formed on red clover roots.

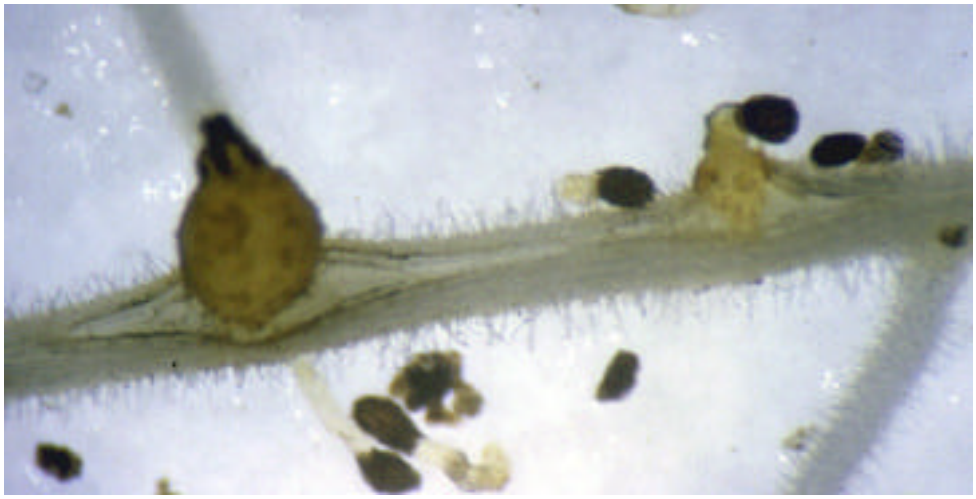

Figure 2.8. Small broomrape (*O. minor*) attachments inducing tissue splits of red clover root cortex.

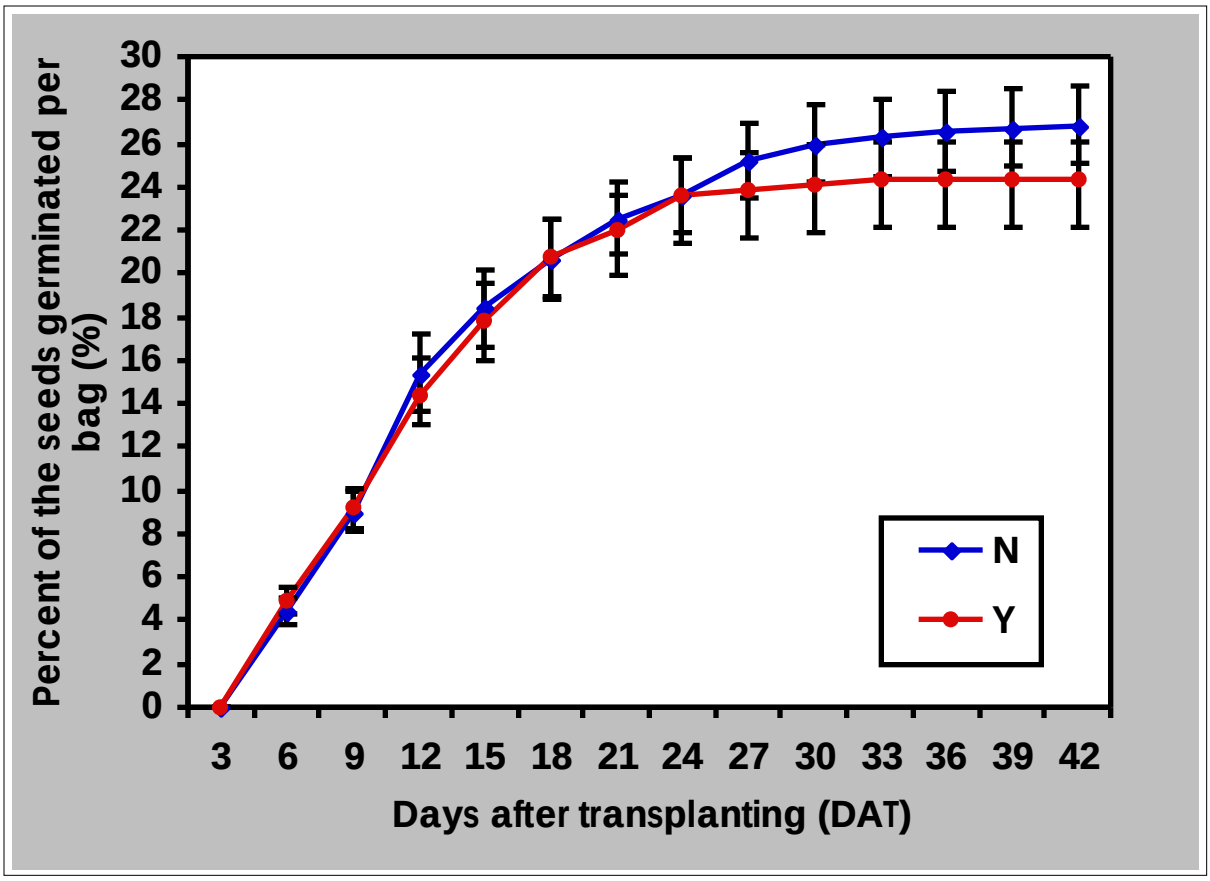

Figure 2.9. Egyptian broomrape (*O. aegyptiaca*) percent germination. Number of germinated seeds were recorded every 3 d for a period of 42 d. N – denotes non-inoculated treatments (without addition of rhizobacteria); Y – denotes inoculated treatments (with addition of rhizobacteria). Data are the means of 30 replications (α = 0.05; SASâ 1989).

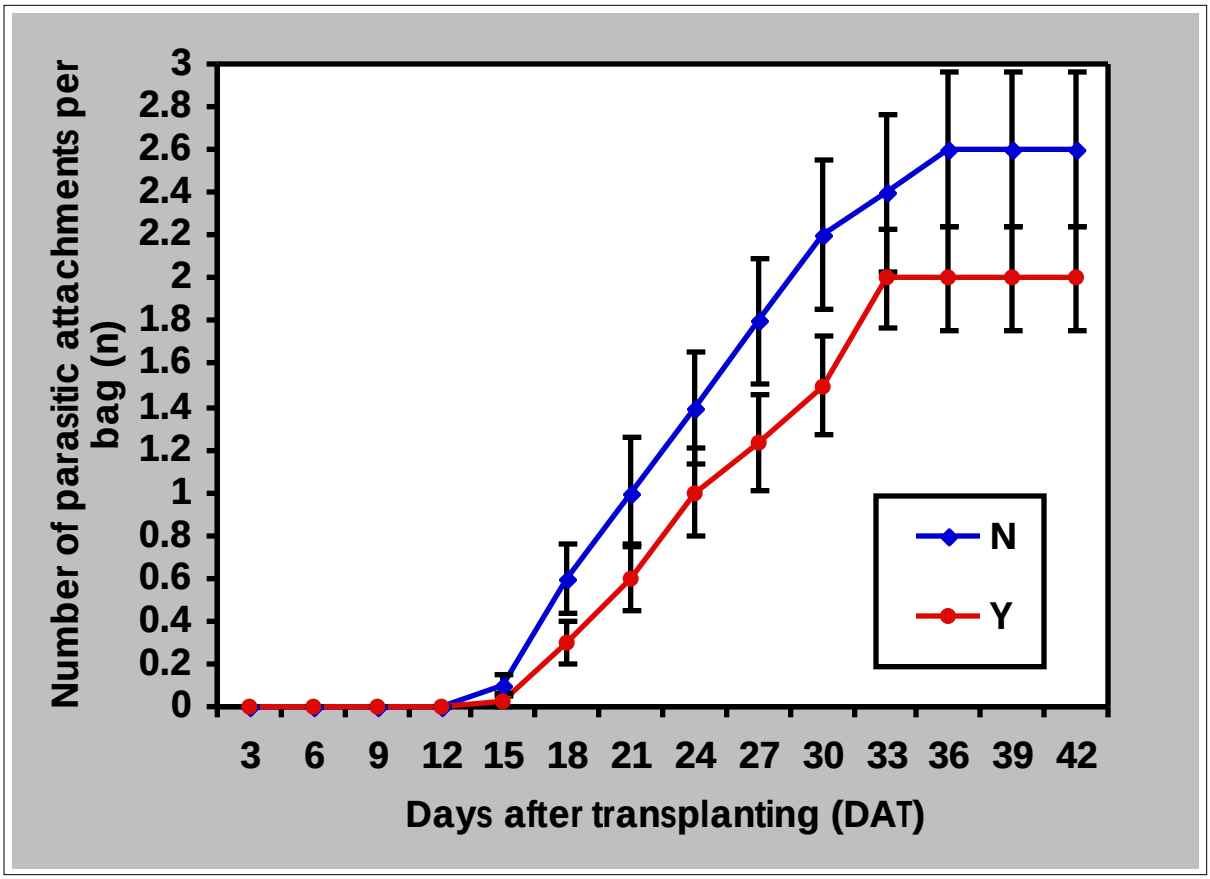

Figure 2.10. Number of Egyptian broomrape (*O. aegyptiaca*) attachments formed on red clover. Number of attachments was recorded every 3 d for a period of 42 d. N – denotes non-inoculated treatments (without addition of rhizobacteria); Y – denotes inoculated treatments (with addition of rhizobacteria). Data are the means of 30 replications (α = 0.05; SASâ 1989).

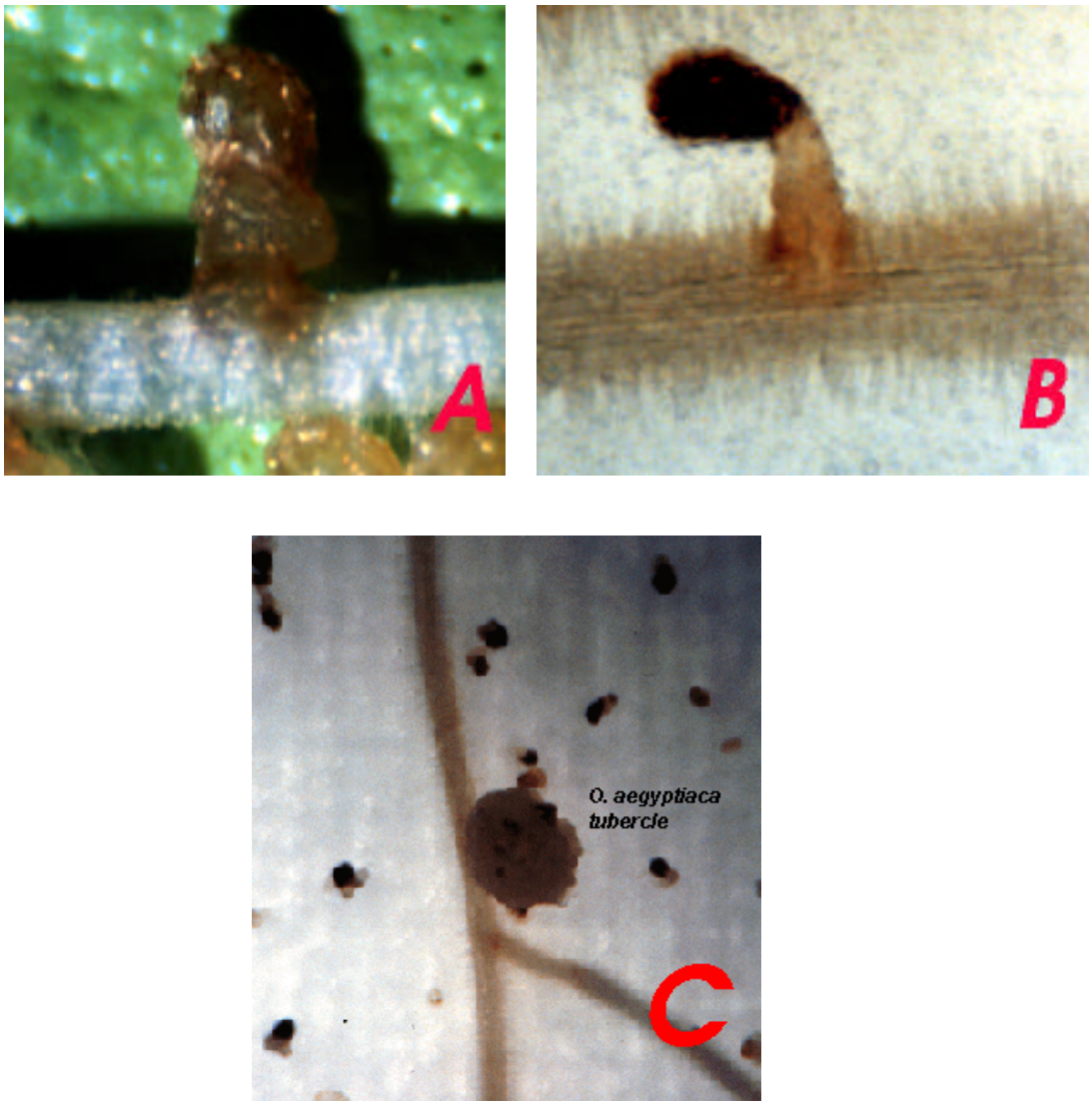

Figure 2.11. Egyptian broomrape (*O. aegyptiaca*) attachments on the root of red clover. A, B - early stages of the attachment; C - developing tubercle.

Figure 2.12. Longitudinal section of the nodule formed on the roots of red clover. Lh - indicates coloration due to *leghemoglobin*.

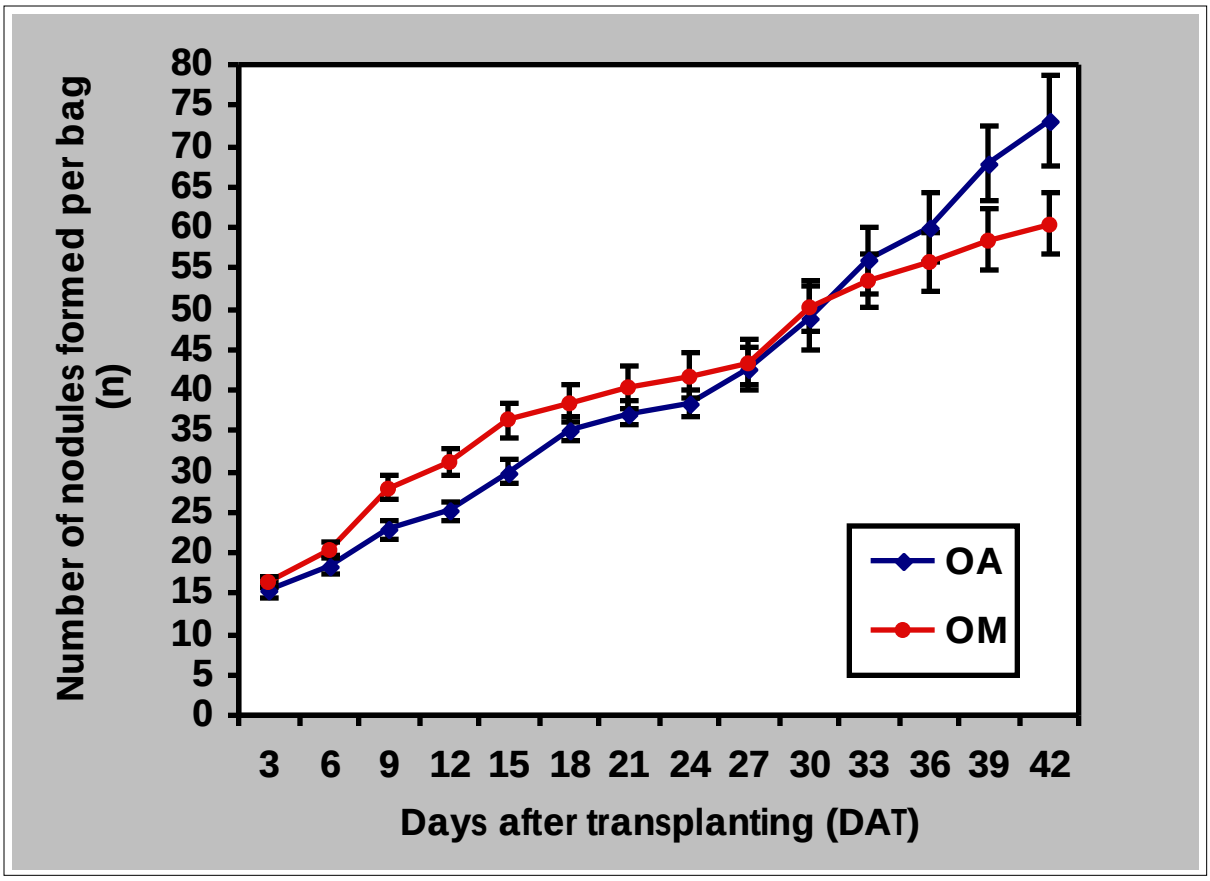

Figure 2.13. Number of nodules formed on inoculated red clover in the presence of *O. minor* and *O. aegyptiaca*. Number of nodules formed was recorded every 3 d for a period of 42 d. OA – denotes observations in *O. aegyptiaca* treatments; OM – denotes observations in *O. minor* treatments. Data are the means of 30 replications (α = 0.05; SASâ 1989).

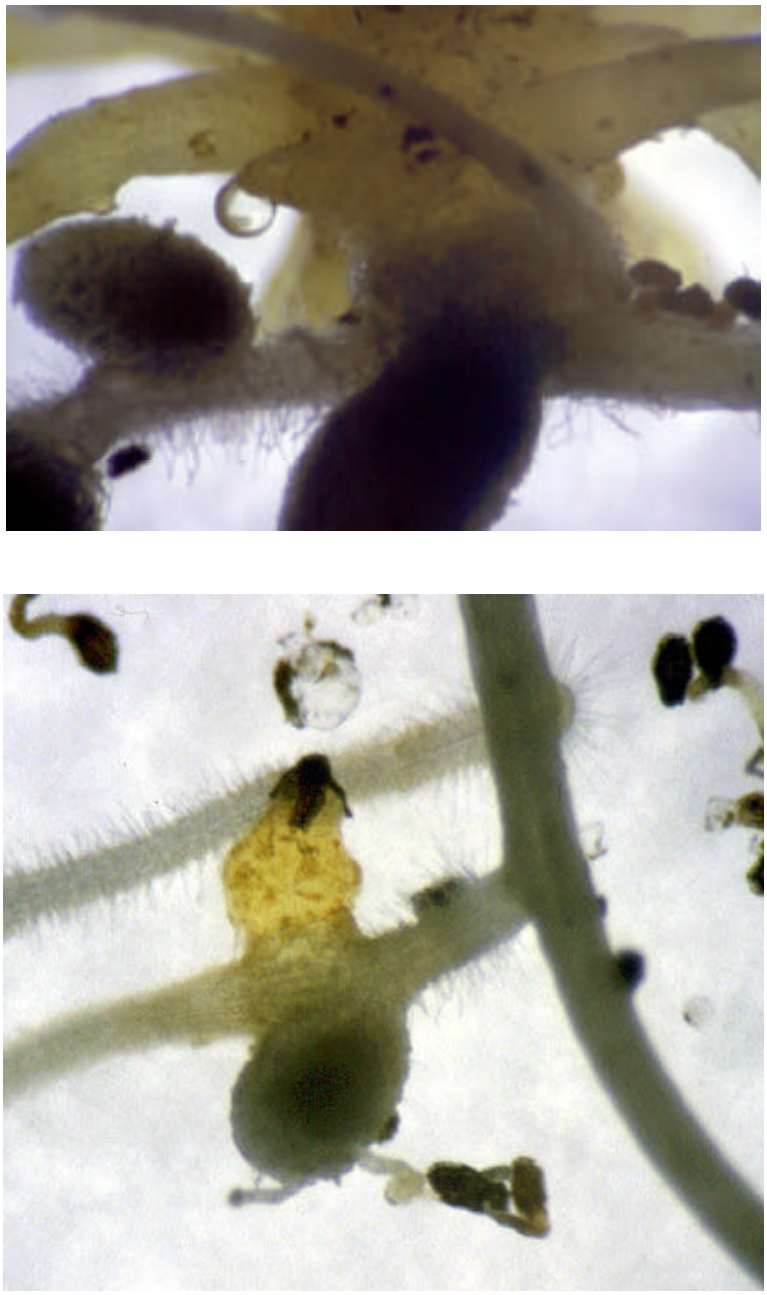

Figure 2.14. Comparison between *O. minor* attachments and rhizobacterial nodules.