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POSTEMBRYONIC DEVELOPMENT OF *CISTANCHE TUBULOSA* (SCHRENK) WHIGT

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Abstract

The embryo of the mature seed of the parasitic angiosperm *Cistanche tubulosa* (Schenk) Whigt remains at pro-embryo stage. Little is known about the establishment of shoot and root apical meristems at poles of the embryonic body during postembryonic development. Thus, structure of the embryo of the mature seed and how and what the shoot and root apical meristems develop into during postembryonic development were investigated using light and dissecting stereo-microscopes. In the mature seed, the plumular pole of the embryo consists of large vacuolated, dense cells, containing starch granules and the radicular pole consists of decidedly smaller and denser cells lacking starch. During seed germination and parasitization, plumular pole cells expired without forming the apical meristem or cotyledons; while radicular pole cells formed a meristematic zone, which developed into a young parasite. We therefore conclude that: (1) the embryo of *Cistanche* consists of a nutrient-storage region (the plumular pole) and a meristematic region (the radicular pole); (2) during postembryonic stages, the plumular pole performs storage function and supplies its stored nutrients as well as those of the endosperm to the radicular pole; the radicular pole was the only origin of the apical meristems forming *Cistanche*. This study provides some useful information on the embryology of the parasitic plant and elucidates the basis for ecological adaptation and evolutionary strategy of this plant.

Introduction

The developmental studies in higher plants deal with by the postembryonic stages. The shoot and root apical meristems are centers of activity during postembryonic development. They give rise to the shoot and root systems by their continued activity. However, the origins of the shoot and root apical meristems differ among various groups of vascular plants. A very important aspect of embryonic differentiation is the establishment of shoot and root apical meristems at approximately opposite poles of the embryonic body (Steeves & Sussex, 1989).

The mature seed of Orobanchaceae contains an embryo which does not have the conventional morphology as it lacks the radicle, hypocotyl, plumule and cotyledons. In a previous work, Kadry & Tewfic (1956a, 1956b) described the seed germination of *Orobanche crenata In vivo*. Upon germination the morphological radicular pole of the embryo formed a "germ-tube-like organ". Its growing tip came into contact with the root of the host and then penetrated it, forming the primary haustorium. That part of the germ tube remaining outside the host root formed a tubercle. Subsequently a single stem was differentiated from the tubercle. Similar observations were made for *O. hederae* (Privat, 1960), *O. aegyptiaca* (Rangaswamy, 1963), and *C. tubulosa* in a tissue culture (Rangan & Rangaswamy, 1968). Interestingly, Kumar (1977) reported that *O. aegyptiaca* seed germination *in vitro* followed two patterns of morphogenesis i.e., monopolar and bipolar. However, these previous workers did not investigate the postembryonic development of *Orobanche* from the meristematic aspect.

Aber & Sallé (1983) attempted to explain the seed germination of *O. crenata* from the meristematic aspect, but they found no direct evidence of cell division in the root apex. Instead, they suggested that the structure of the developing root apex gave an indirect evidence of meristematic activity that gave rise to root elongation. Joel & Losner-Goshen's (1994) observations on *O. cummana* and *O. aegyptiaca* were in conformity with those on *O. crenata*, but they also found no direct evidence of cell division in the root apex. They presumed also from the cell structure of the developing root apex that the developing root apex had a meristem. Although Ben-hod *et al*., (1991) found no direct evidence that cells of the radicular pole of *O. aegyptiaca* were dividing, but stated that the radicular apex had an apical meristem.

Previous workers studied the development of the radicular pole from the meristematic aspect, however, they only presumed a meristematic activity in the radicular pole during postembryonic development stages. No systemtic investigation of the function of meristematic activity during these stages was carried out. Thus there are yet no reports on the establishment and function of the apical meristem of the plumular pole during postembryonic development. Since the 2000s, Orobanchaceae has been studied by numerous investigators, especially with regard to factors effecting germination, but in spite of the importance of their postembryonic development in determining the parasitic course, much remains to be done (Bar Nun *et al*., 2003; Daisuke *et al*., 2003, 2005; Song *et al*., 2005, 2006; Kaori *et al*., 2007, Mir *et al*., 2009).

Cistanche tubulosa (Schenk) Whigt, a member of Orobancheace, is an important traditional Chinese medicine and is used for the treatment of kidney problems and neurasthenia (Ihsan *et al.*, 2010). In order to domesticate the wild *Cistanche*, the artificial cultivation of *Cistanche* was studied. During the domestication, we wondered how *Cistanche* seed developed into a young plant from the establishment of shoot and root apical meristems at poles of the embryonic body i.e., how and what the shoot and root apical meristems develop into. Our aims in this work were to investigate the establishment of shoot and root apical meristems at poles of the embryonic body during postembryonic development and determine how the meristem develops into a young parasitic plant.

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Materials and Methods

Plant material and growth condition: *Cistanche tubulosa* seeds were collected from *Chinese tamarisk* Twing fields in Sinkiang Province of China in 2005. Seeds with diameter greater than 0.7mm were used for the study. Seeds were double surface-sterilized by sequential immersions in 70% ethanol for 1 min., and 0.1% Sodium hypochlorite containing 0.1%Tween-20 for 15 min., (Zhou *et al*., 2004) and then placed in a sterile 9 cm Petri dish lined with two layers of filter paper wetted with 6 ml of 10mg.L^{-1} fluridone (Sigma-Aldrich Laborchemikalien GmbH, Seelze, Germany) solution. The Petri dishes were sealed with Parafilm and wrapped with aluminum foil to provide absolute darkness. These were then placed in dark, controlled growth chamber with temperature set at 25°C for germination (Wang *et al*., 2006). Seedlings were considered to be in the earlier stage when the radicle emerged from the seed testa. When the length of the emerging radicle was equal to or longer than twice its length, seedlings were considered to be in the late stage. Seedlings in both earlier and later stages were fixed in FAA (70% ethanol:acetic acid:formaldehyde, 18:1:1, by vol.).

In order to obtain young parasite plants at various developmental stages, one gram of seeds were evenly sown in a potted sandy soil containing two-year-old *Chinese tamarisk* (host plant) with well developed roots. Six months after planting, the host plant and sandy soil were carefully removed from the pot and on host roots containing parasitizing young Cistanche of different sizes were fixed in FAA.

Light microscopy: An examination of *Cistanche* seedlings was carried out with the aid of a dissecting stereo-microscope (Olympus SZH10, Japan). Mature seeds, seedlings and young *Cistanche* fixed in FAA at various developmental stages were then dehydrated in a graded ethanol series, embedded in paraffin and 8 µm sections taken. Sections were stained either with safranine and fast green or with Haidenhain's iron-alum haematoxylin and observed with the light microscope (Olympus BX51, Japan).

Results

The seed: The globose seeds had a hard testa and enclosed a small embryo surrounded by endosperm (Fig. 1A). The subglobose embryo lacked organization into radicle, hypocotyl, cotyledon, and plumule (Fig. 1B). The pole of the embryo proximal to the micropyle is the morphological radicular pole and that distal to the micropyle is the morphological plumular pole. The plumular pole of the embryo consists of large, vacuolated, dense cells containing starch granules and the radicular pole consists of decidedly smaller and denser cells lacking starch (Figs. 1B-D).

Germination: The radicular pole emerged from the seed coat was devoid of a conductive tissue and a root cap. The central cells of the radicular pole are relatively small and nearly isodiametric. Their walls, mostly set at right angles to each other, overlap closely, leaving no spaces. The lumen is filled with dense protoplasm, with a relatively large nucleus (Fig. 2A). The cells at the plumular pole enlarge, elongate and become vacuolated (Fig. 2B). Starch was detected easily in the cells of plumular pole and the peripheral cells of the radicular pole with the aid of a polarized-light microscope (Figs. 2B-C), but the central cells of the radicular pole were devoid of starch (Fig. 2A).

Along with the radicular tube (the emerging part of the radicular pole from the seed coat was called the radicular tube) extension, cells at the plumular pole and the axial cells between these two embryonic poles enlarged and formed elongated, highly vacuolated, parenchyma cells (Figs. 2D-E). The central cells of the radicular pole included dense protoplasm and a relatively large nucleus (Fig. 2F). Cells of the plumular pole and endosperm were initially high in starch, but the content decreased as the tube elongated (Fig. 2D). The peripheral cells of the radicular pole and the axial cells between these two embryonic poles were initially low in starch, but the content increased as the tube elongated (Figs. 2E-F). However, the central cells of the radicular pole were still devoid of starch (Fig. 2F).

Formation of a haustorium: During later stages of the germination, in which the central cells of the radicular pole further divided and vacuolated, the apex of the radicular pole became swollen (Fig. 3A). It was easily detected that some central cells of the radicular pole were dividing at that stage. Each nucleus had two or more nucleoli (Fig. 3B). The swelling apex became spherical. This spherical organ was the haustorium (Fig. 4). However, the central cells of the radicular pole still retained dense protoplasm and a relatively large nucleus (Fig. 5).

The parasite: In the absence of host root, the development of the haustorium stopped at the conical stage. However, with host contact, field and laboratory studies revealed large numbers of haustoria that fail to make functional attachments. As soon as the haustorium was attached to the host root, the penetration of the host started. First, the peripheral cells and the central cells of the radicular pole reached the cortex cells of host root. At that stage, the meristematic zone of the radicular pole rapidly proliferated and differentiated, and parasite vessels formed (Fig. 6A). Then, differentiation of the axial xylem strand was established, connecting to the host root xylem, and developing acropetally in the haustorium. The cells near the axial xylem strand were still devoid of starch as examined under polarized-light microscope (Fig. 6B).

Once the parasite was established, the radicular tube gradually began to atrophy (Fig. 7A). If the radicular tube was gently removed from the tubercle, the place covered by the radicular tube had a lump (Fig. 7B). The radicular tube was empty and nutrients in the endosperm were exhausted. The lump consists of parenchyma cells. Cells underlying the lump were also parenchyma and not meristematic cells (Fig. 8A) and had conspicuous starch; however, cells of the meristematic zone were devoid of starch (Fig. 8B).

Plant parasite: To further confirm the relationship of the meristematic cells with the starch, the transverse section through the shoot apical meristems of the young parasite plant was made. Cells at the growing point were also devoid of starch; however, other cells had conspicuous starch (Fig. 9).

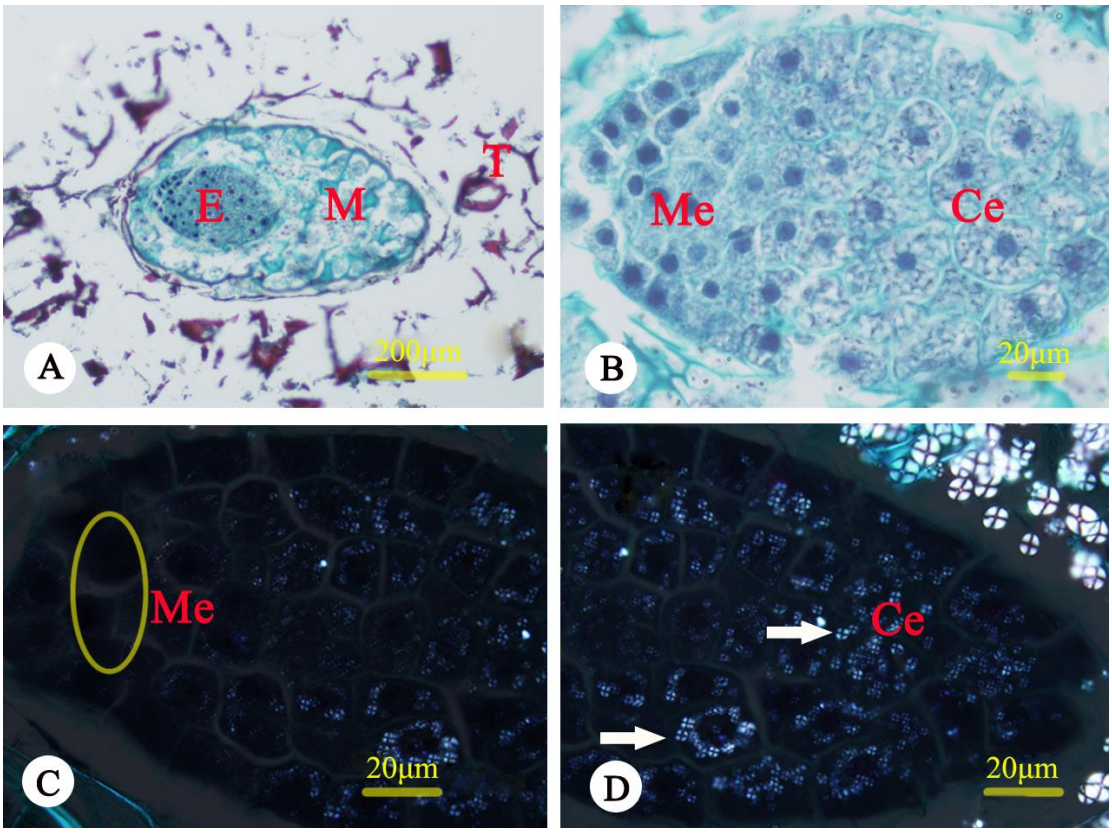

Fig. 1. Light micrograph of longitudinal sections of *Cistanche tubulosa* seed. A, General image showing the globose seeds have a hard testa and enclose a small embryo surrounded by endosperm. B, Enlargement of Figure A. Cells at the chalazal end are large, vacuolated and have dense cytoplasm while those at the micropylar end are decidedly smaller and have denser cytoplasm. C, Polarized-light micrograph showing cells at the micropylar end are devoid of starch grains (yellow oval). D, Polarized-light micrograph showing cells at the chalazal end have starch granules (arrows). Ce-chalazal end, E-embryo, M-endosperm, Me-micropylar end, T-testa.

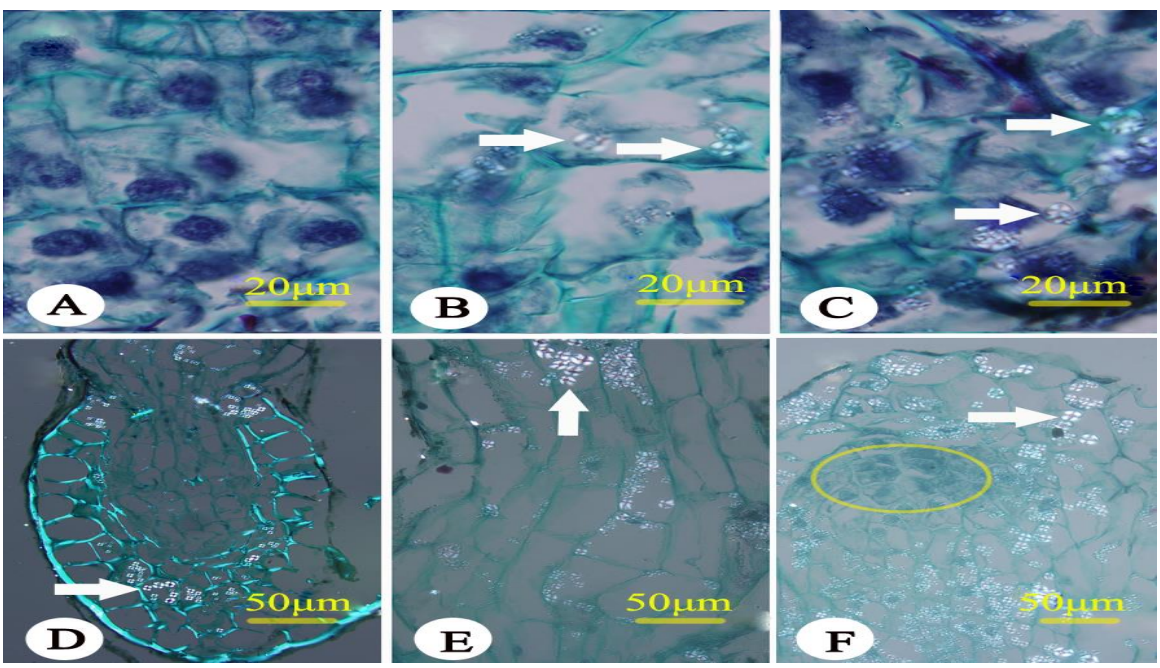

Fig. 2. Polarized-light micrograph of longitudinal sections of the different parts of *C. tubulosa* seedling in the earlier and later stages. A, Central cells of the radicular pole in earlier stages. Central cells of the radicular pole are relatively small and nearly isodiametric and have dense protoplasm, with relatively large nucleus. Their walls are mostly set at right angles to each other. These cells have no starch. B, Cells of the plumular pole in earlier stages. Cells at the plumular pole enlarge, elongate, become vacuolated and have starch (arrows). C, Peripheral cells of the radicular pole in earlier stages. Cells of the radicular pole have starch (arrows). D, Endosperm cells and the plumular pole cells in the later stage. The starch content of the endosperm and the plumular pole decreasing with the elongation of the radicular tube (arrow). E, The middle part of the radicular tube. The starch content of the middle part of the radicular tube increasing with elongation of the radicular tube (arrow). F, Tip of the seedling showing the peripheral cells of the radicular pole have starch and the center cells of the radicular pole are devoid of starch (arrow).

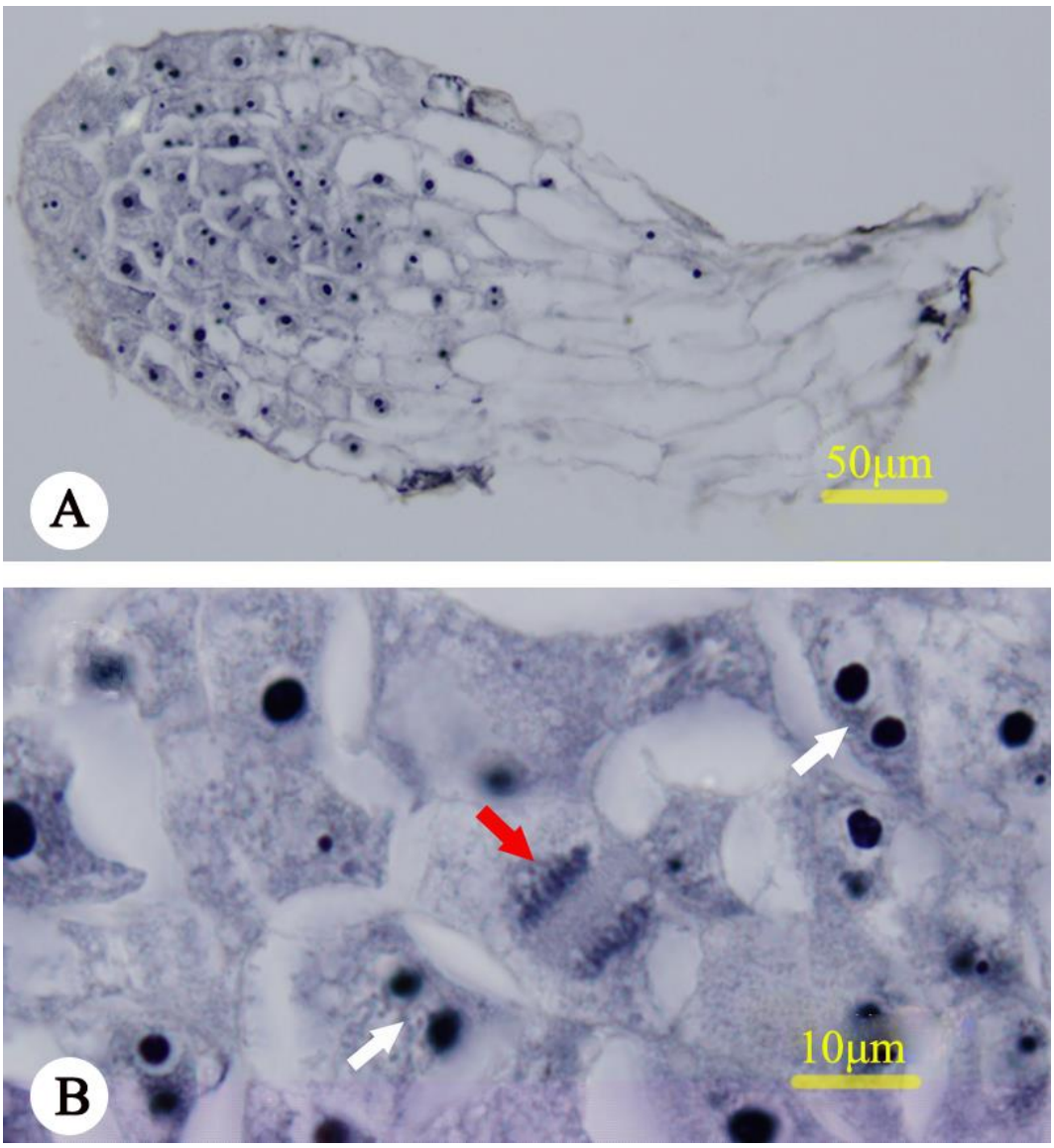

Fig. 3. Light micrograph of longitudinal section through the radicular tube tip of *C. tubulosa* seedling. Haidenhain's iron-alum haematoxylin staining. A, Cells arranged in longitudinal rows with basipetal gradients of cell elongation and cell vacuolation. B, Enlargement of Figure A. Each nucleus has two or more nucleoli (white arrows). Some cells are dividing (red arrow).

Fig. 4. Light micrograph of *C. tubulosa* seedling attached to a host root. The conical radicular tube tip becomes spherical (arrow). Hr-host root.

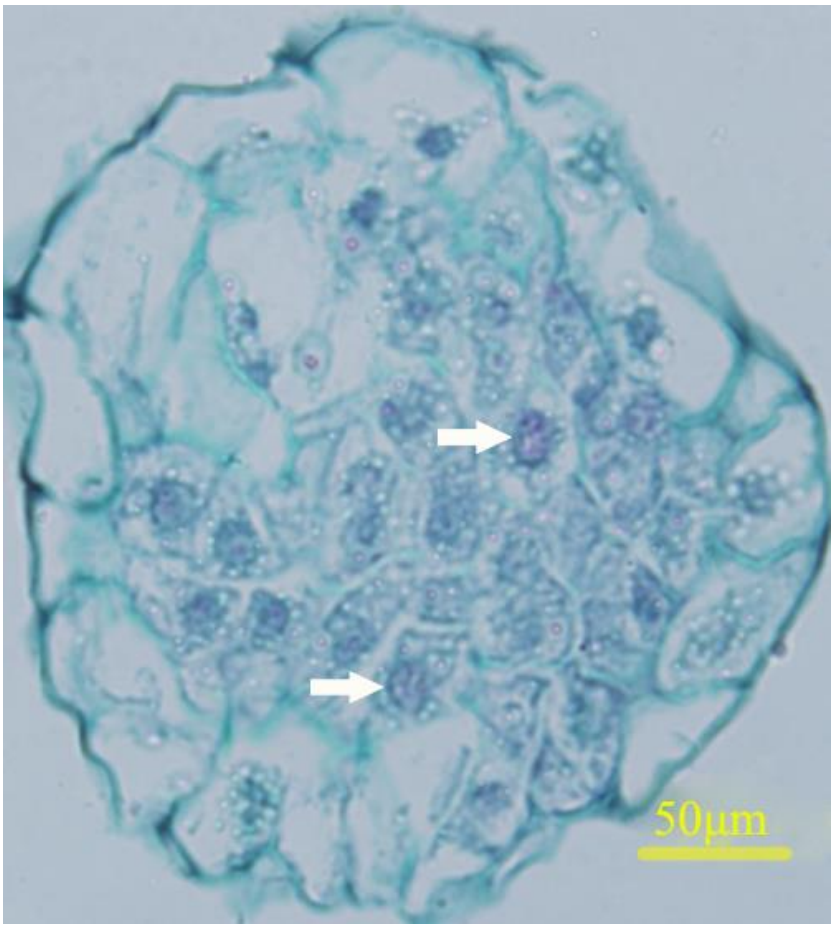

Fig. 5. Light micrograph of cross section of the middle part of *C. tubulosa* haustorium. Central cells of the haustorium have dense protoplasm and large nuclei (arrows) and the peripheral cells of the haustorium are vacuolated and enlarged.

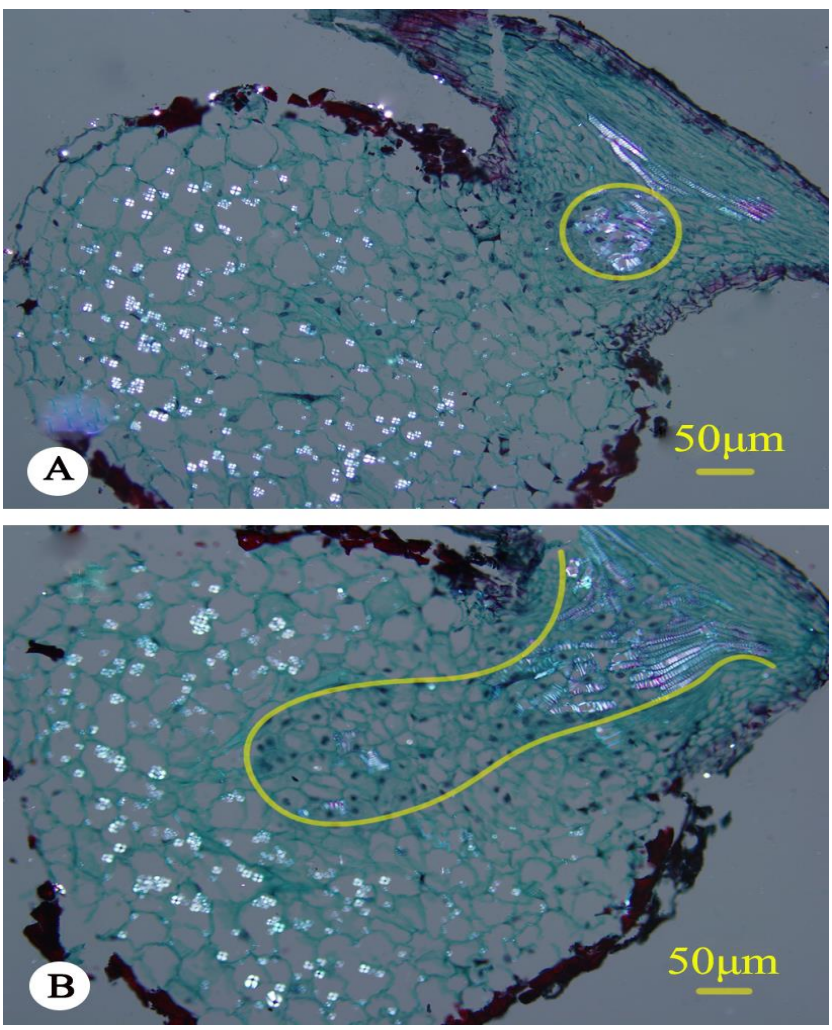

Fig. 6. Polarized-light micrograph of longitudinal section of the meristematic zone of the tubercle of *C. tubulosa* and the attached host root during the earlier and later stages of the parasite.

A, Early stage of parasitization, the haustorial xylem enters the cortex of the host root. Cells of the meristematic zone begin to differentiate and the vessels of the young plant form. The yellow circle indicates the meristematic zone. B, Late stage, the haustorial xylem reach the stele of the host root, showing the xylem connection between the parasite and the host root; the meristematic zone elongated acropetally in the haustorium. Yellow line delimits the meristematic zone from the non-meristematic zone.

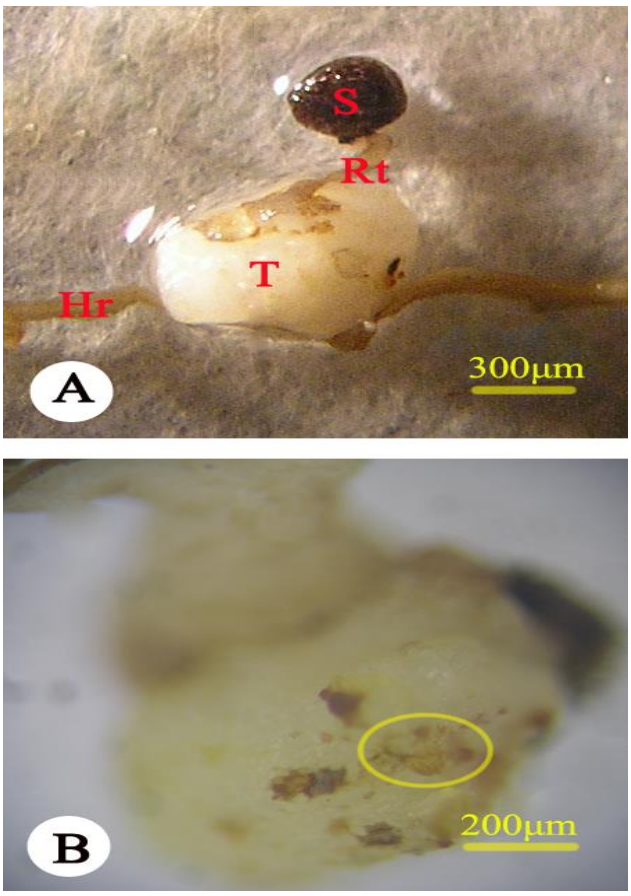

Fig. 7. Dissecting stereo-microscope micrograph of the young *C. tubulosa* plant. A, The radicular tube becomes atrophied. B, Point in which the radicular tube cover has a lump. The yellow oval indicates the lump. Hr-host root, Rt-radicular tube, S-seed, T-tubercle.

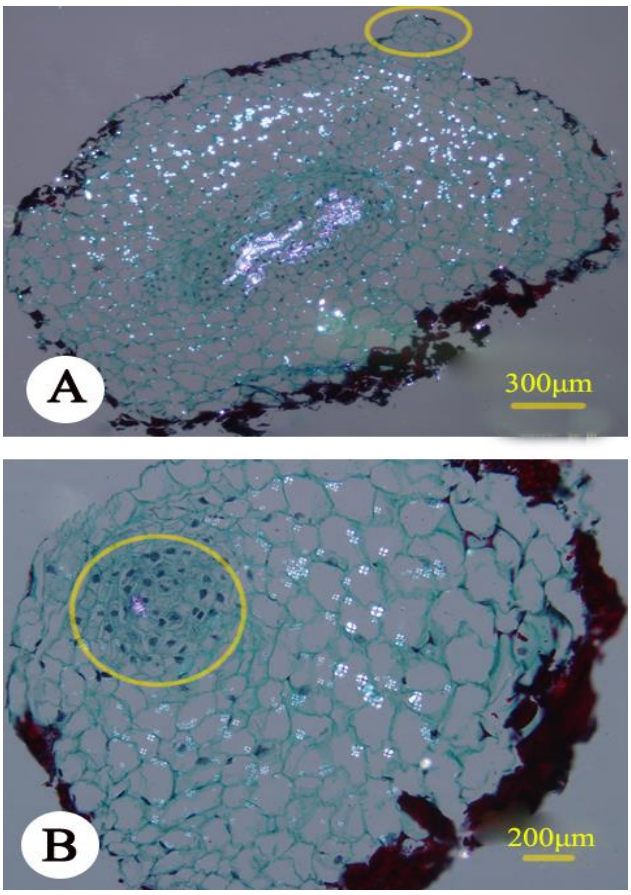

Fig. 8. Light micrograph of sections of the young *C. tubulosa* plant. A, Longitudinal section through the lump of the tubercle showing there is no meristematic zone underlying the lump. The yellow oval indicates the lump. B, Transverse section of the middle part of the tubercle showing there is only a meristematic zone in the tubercle. The yellow circle indicates the meristematic zone.

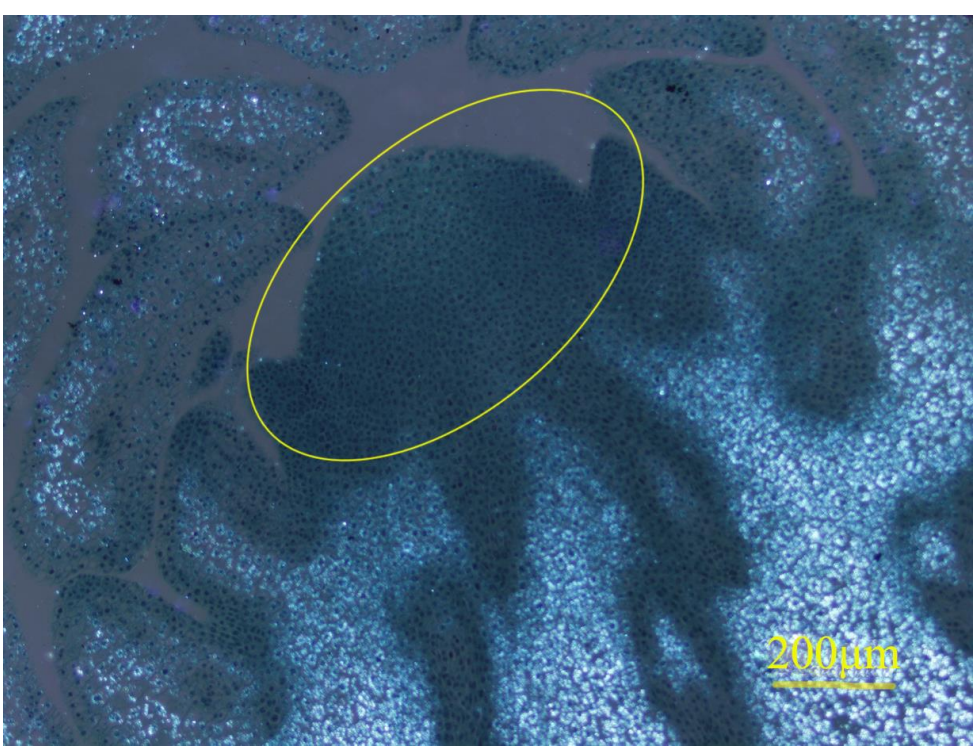

Fig. 9. Light micrograph of the cross section of shoot apical meristem of *C. tubulosa* showing that cells of the meristematic zone are devoid of starch. The yellow oval indicates the meristematic zone.

Discussion

The plumular pole had no morphological differentiation during seed germination and parasitization as reported by Joel & Losner-Goshen (1994) for *O. cummana* and *O. aegyptiaca*. For *O. crenata*, Kumar (1977) reported that this morphogenic suppression could be chemically removed to allow the plumular pole to produce a shoot bud. The removal of the morphogenic suppression may be the result of hormonal regulation. The absence of morphological differentiation in the plumular pole was reflective of the parasitic characteristic of *Cistanche*. The possible reason for the inability of the plumular pole to form cotyledons was that *Cistanche* is a holoparasitic plant, i.e., its nutrition is completely dependent on the host.

The plumular pole did not produce a meristematic zone and finally expired during seed germination and parasitization. However, it became an "organ" for storing nutrients and absorbing endospermous nutrients, supplying it as well as endosperm nutrients to support the radicular pole forming a haustorium. The failure of the plumular pole to produce a meristematic zone differs from the observation of Joel & Losner-Goshen (1994) for *O. cummana* and *O. aegyptiaca* in which some cells of the plumular pole neither divided nor grew during germination but maintained a dense cytoplasm. This difference might be because: 1) the authors' observation was at the earlier germination stage; 2) *Orobanche* and *Cistanche* have different postembryonic development processes. The nutrients in cells of both the plumular pole and endosperm were transferred to the radicular tube as it elongated. This observation was consistent with that of Kumar (1977) on *O. crenata*. In addition, we noticed that cells of the plumular pole were the only link between the embryo and endosperm and the starch content of cells of the plumular pole and endosperm decreased as the radicular tube elongated. The starch content of the radicular tube also increased as it elongated and once the parasite was established, the radicular tube gradually atrophied due to exhaustion of nutrients in the endosperm and plumular pole. The results could prove that cells of the plumular pole absorbed endospermous nutrients and transferred them to the radicular pole.

The plumular pole was transformed into an "organ" for storing and absorbing nutrients instead of a meristem, which was beneficial to the parasitic activity of *Cistanche*. *Cistanche* seeds are very small, weighing only 0.07g per thousand seeds, and measuring circa 0.8 mm in length and 0.4 mm in width. Their endosperm stores limited nutrients. The plumular pole with the function of storing and absorbing nutrients ensured the essential energy for embryo development and decreased the energy for producing a meristematic zone. Thus, the development mode of the plumular pole could increase the parasite opportunity of *Cistanche*, which is of strong ecological significance.

The radicular pole developed into a meristematic zone during seed germination and parasitization. Previous workers only presumed that the radicular pole had a meristematic zone (Aber & Sallé, 1983; Joel & Losner-Goshen, 1994). We found a direct evidence that cells at the radicular pole were dividing. A systematic study of the development process of the meristematic zone showed that the activity of this zone initiated germination, haustorium formation and growth of the young parasite plant. This proved that the radicular pole was the only source of the apical meristems forming *Cistanche*.

Plastids were less well differentiated in the central zone of the meristem than in the peripheral regions (Steeves & Sussex, 1989). From seed to *Cistanche* young plant, the absence of starch in the central cells of the radicualr pole and its presence in the plumular pole suggested that the radicular pole was a meristematic region while the plumular pole was not.

The *Cistanche* embryo was divided into nutrient-storage and meristematic regions based on the classification of Orchid embryo (Arditti, 1967). Unlike that of *Cistanche*, the embryo of Orchidaceae consisted of a food-storage region (the radicular pole, previously termed in the literature "anterior region") and a meristematic region (the plumular pole, previously termed in the literature "posterior region"). The plumular pole completed seedling formation (Carlson, 1943; Arditti, 1967; Kumar, 1977).The classification method for Orchid embryo was appropriate for the Orchid seed which lacks an endosperm. Interestingly, the embryo of *Cistanche* seed which has an endosperm also performs the storage function. This feature might be related to the parasitic property of *Cistanche*.

In conclusion, the plumular pole functions in storage and absorption of nutrients, supplying same to support the development of the radicular pole; the radicular pole was the only source of the apical meristems forming the *Cistanche* plant. This study provided some useful information on the embryology of the parasitic plant and elucidated the basis for ecological adaptation and evolutionary strategy of the parasite plant.

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