Microscopic visualization of *Peronospora variabilis* Gäum., the cause of quinoa downy mildew in plant tissues at different stages of plant growth

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ABSTRACT---*Peronospora variabilis* Gäum., the cause of quinoa downy mildew (DM) was visualized into various plant organs by the aid of compound microscope. The fungal oospores could be detected very early after seed germination. They were observed in the pith of radicles (after 3 days) and the cortex of hypocotyls as well as the mesophyll of cotyledons (after 7 days). As noticed after 15 days of planting, oospores started to germinate in the cortex of hypocotyls and mesophyll of cotyledons initiating the fungal mycelium into the juvenile seedling. Gametangia were observed in the leaf mesophyll 45 days sowing. Fungal mycelium, however, continued to be seen in different tissues; in perisperm of the developing seed at 80 days and in the root conjunctive parenchyma at 120 days of plant growth. The fungal oospores became apparently visible in all tissues of quinoa plants from 45 days up to 120 days after planting.

Key words : *Peronospora variabilis*, quinoa, downy mildew, oospores, gametangia, hypocotyls, cotyledon, mesophyll, perisperm, parenchyma.

1. INTRODUCTION

Quinoa (*Chenopodium quinoa* Willd.), an ancient food crop of the Andean Region of South America, was recently introduced as a new promising winter food crop to Egypt in 2017. Its good growth in poor, drought and saline soils provides quinoa for high production in developing countries. The crop, thereby can play a key role in food production in new reclaimed lands of Egypt. The biggest constraint in quinoa production all over the world is the infection with downy mildew (DM) caused by *Peronospora variabilis* Gäum (formerly *P. farinosa* f. sp. *chenopodii* Byfor). Downy mildew was reported to cause losses in the yield production as reported at 33-99 % (Danielsen *et al.*, 1999). The disease has been reported for the first time as a devastating disease of quinoa from Egypt in 2014 (El-Assiuty *et al.*, 2014).

Epidemics of this type of diseases are associated, particularly, with the primary infection by either oospores or asexual spores. Oospores presented under the pericarp of quinoa fruits were reported as the initial source of inoculum (Danielsen *et al.*, 2004 and Kitz, 2008). In accordance with that, oospores and other fungal structures of *P. variabilis* were shown by El-Assiuty *et al.* (2019) to be located in different parts of quinoa seed. In depth study was done to follow up the passage of *P. variabilis* inside plant tissues at different stages of growth. This is of great importance to appoint the appropriate method and the proper time of application to control the disease.

2. MATERIALS AND METHODS

Seeds of a quinoa variety, sensitive to infection with DM; c.v. Misr-1were used in the present investigation. Otherwise stated, seeds were planted in potted-soil in Oct. 2018 in Plant Pathology Research Institute, ARC, Giza. Twenty five clay pots (capacity about 2.5 kg soil) contained air-dried autoclaved-sieved-clay loamy soil were used. Pots were planted with surface sterilized (with 1% NaOCl) quinoa seeds and maintained in greenhouse at 18-20ºC and 85-95 % RH. After two weeks of planting, thinning was performed to 5 seedlings per pot and irrigation and fertilization were made as usual. Resulting plants were periodically uprooted for investigation starting from 15 days of planting. Anatomical performance of juvenile plantlets younger than 15 day-old (at 3 and 7 days of seeding) was done to sprouted seeds prepared by blotting into sterilized wet cheese cloth. As onset of the disease symptoms often seen late in the season (about 45 days of planting), plants were randomly taken for the anatomical study regardless of the signs of infection (whether symptomatic or asymptomatic).

Histopathological investigation of different quinoa plant organs

To identify the occurrence of fungal structures of the target DM pathogen in different plant organs, 15 samples were periodically taken randomly starting from 3 days after seed germination up to 120 days of sowing. Of each plantlet (up to 15-day old seedling), radicles, hypocotyls and cotyledons were separated. From elder plants, however segments (1-2 cm long) were taken from the principal main roots, upper fifth internodes of stems, leaf lamina and petioles of the upper fifth node of the principal axis and the basal part of the main inflorescence. The developing fruits were selected randomly from the first basal secondary axis of the main inflorescence on the 80-day old plants. Sterilized sharp razor blade or scissors was used in this process. Samples were fixed in 70% ethanol and ten segments of each plant part were

randomly obtained from the fixed plant material for investigation.

A-Clearing of the whole mounts and cross sections

Processing of clearing was made by soaking samples under investigation in 25 ml of 1 M KOH for 30 min. at 90ºC to hydrolyze the protoplasmic contents in order to make the tissues opaque needed for the microscopic examination (Danielsen *et al.* , 2004). Thereafter, samples were rinsed in water, acidified with dilute HCl and stained with lactophenol-trypan blue (10 ml lactic acid, 10 ml glycerol, 10 g phenol, 10 mg trypan blue dissolved in 10 ml water) according to Keogh *et al.* (1980) and Koch and Slusarenko (1990). The segments and cross sections were then washed in chloral hydrate to remove the excess stain, mounted in the same solution on clean glass slides and gently pressed. The KOH-treated tissues were viewed and photographed under a compound microscope. Segments of roots of 120 day-old plants were cleared in 80% ethanol in glass tubes in boiling water-bath, followed by 4% sodium hypochlorite (NaClO), free hand sectioned and finally stained with lactophenol cotton blue (Tisdall and Donnelly, 1988). In some cases, standard free-hand sections were placed in clearing solution of lactic acid (85-92%) saturated with chloral hydrate (Lux *et al.*, 2005). These sections were stained in lactophenol-cotton blue or trypan blue.

B- Cross sectioning of the paraffin-embedded plant materials

The fixed plant segments and the developing seeds were dehydrated following standard methods, embedded in Paraplast, sectioned in transverse directions at 5 µm, and mounted on glass slides. The Paraplast was removed from the slides with xylene. The deparaffinized glass slides were then washed in descending graded series of ethanol (from absolute ethanol to 70% in water), brought into water, and finally stained with lactophenol-trypan blue (Koch and Slusarenko, 1990). Because of the difficulty to localize the trypan blue-stained intercellular fungal structures in the paraffin sections due to the dense cell inclusions of the perisperm and endosperm of seed (Prego *et al.*, 1998), staining with Grocott's Methenamine silver stain (GMS) was followed as described by Grocott (1955). The principle of this method of staining is that chromic acid oxidation forms aldehydes from the components of fungal cell wall polysaccharides and subsequently demonstrated by reduction of an alkaline hexamine-silver complex (cf. Luna, 1992). By this method of staining, the fungal structures will be stained black.

2. RESULTS AND DISCUSSION

Results (Figs. 1-7 &Table 1) show that the fungal structures started to appear in examined tissues very early as oospores in the pith of radicle after 3days of seed germination (Fig.1A). It should be notified that this unexpected appearance of *P. variabilis* within quinoa radicle just after seed germination was supported by findings of Taha (2019). He could prove by PCR assay the presence of the pathogen in tissues of radicle just after seed germination. In 7 and 15-day old seedlings, only oospores were found in the cortex of the hypocotyl and in mesophyll of the whole mount of cotyledon (Fig.2 a & B & Fig.3 a& B). As shown in Figs. (2 B & 3 C), oospores could germinate in quinoa seedlings after 15 days of planting. Early appearance of oospores of *P. variabilis* as they strictly visualized in the present study could be possibly attributed to their presence in the embryonic seed tissues as recorded by El-Assiuty *et al.* (2019). In accordance with these results, we hypothesize that fungal oospores in pith tissues of the germinated seed may act as initial source of spreading mycelium in root tissues (Fig.1 B, C, D& E & Table, 1) as well as in the hypocotyl (Fig.2 B) and the stem (Fig. 2 C, D& E). This finding draws attention to search for critical method of application earlier in the season to prevent the germination of fungal oospores in seedlings.

It was found that diameters of oospores in the current study ranged between 12-30 µm (Table,1), slightly narrower than those found by El-Assiuty *et al.* (2019) and those found by Danielsen *et al.* (2004) who reported that the diameters of oospores ranged between 39-50 µm. Also, Choi *et al.* (2008) measured oospores of *P. variabilis* in leaves of *C. album* at 22.5-32.5µm. The differences in oospore sizes may be attributed to climatic conditions, spore maturity, the organ tissues, fungal strains..… etc.

Examinations at older plant growth revealed that the fungal hyphae were the prevailing structure within different plant tissues. Mycelium started to appear obviously in tissues of 33 day-old plant roots (Fig. 1C) and continued to be seen in plant tissues of stem and leaf midrib up to 120 day-old plants (Figs. 2 C, D, E & 4 A). Less appearance of the gametangia was observed into the examined tissues, where they were precisely seen along with oospores in the leaf mesophyll at 45 days of planting (Fig. 5 B). Oospores were found to have the efficient way to germinate giving two of un-branched germ tubes (Fig. 2B & 3C). It is known that oospores of fungal oomycetes differently germinate by producing single germ tube as in *Sclerospora sorghi* (Pratt, 1978) or two germ tubes as in *Albugo candida* (Verma and Petrie, 1975).

As summarized in Table 1, oospores were found in the mesophyll tissues of the cotyledons at the ages of 7- and 15-day old seedlings (Fig. 3 A, B & C), leaves of 45, 80 and 120-day old quinoa plants (Fig. 4B, C & D), and the mesophyll of the perianth of the developing seed (Fig. 7A). The pathogen was seen as mycelium in the intercellular spaces of the leaf midrib of 80-day old plants (Fig. 4 A), and as oospores in the parenchyma tissues in the petiole of 120-day old plants (Fig. 4 C). Based on the phenological growth stages of quinoa (Sosa-Zuniga *et al.*, 2017), cotyledons, foliage leaves and seeds enclosed by their perianth spontaneously shed away at different times from mother plants. Cotyledons, however started to fall from the principal stems of the seedlings at early stages, whereas at the stages of fruit ripening and plant senescence, dead leaves and stems along with the loosen seeds fall from the mother plants. As stated above, it is suggested that *P. variabilis* possibly survives as resting oospores in the fallen cotyledons, leaves and the perianths of seeds. Accordingly, we hypothesize that the parts shed during the life cycle of quinoa plants may play a role of persistence of oospores in soil and perhaps infect the radicles of quinoa seedlings next season. As *P. variabilis* could infect some weeds as *Chenopodium album* (Choi *et al*., 2010) and *C. murale* (Baiswar *et al.*, 2010), these weeds could be an additional source of infesting field soil with persisted oospores. In Egypt, both *C. album* and *C. murale* are widespread as annual weeds (Boulos and El-Hadidi,1989) and may be considered alternative hosts for DM. From this point of view, the probability of infecting quinoa by resting oospores in soil through the radicle of seedlings or the root system deserves further investigation.

Based on the presence of oospores in the radicle (Fig. 1 A), and on the occurrence of fungal structures in different tissues of quinoa plants (Figs. 2-7 & Table, 1), it is conceivable that after the germination of oospores in the radicle tissues, the formed mycelium spreads acropetally through intercellular spaces of the hypocotyl, and upwards through the aerial branches of the plant until it reaches the leaves, inflorescences, flowers, and finally penetrates the developing seeds. It was observed that few numbers of the fungal structures were found in tissues of collenchyma as shown in Fig. (2 C). this may be attributed to the wall thickenings which impede the extension and growth of fungal hyphae. This hypothesis is supported by the findings of Kitz (2008) who reported that the intercellular mycelium in the mesophyll cells of quinoa leaf infected by DM pathogen was thinner than that in the intercellular spaces of the collenchyma cells in cortex of the leaf petiole. He supposed that the intercellular mycelium in the collenchyma tissue was exposed to relatively high resistance due to the thick cell walls compared to the thin walls of the palisade cells in the leaf lamina.

It is worth mentioning that unlike some previous histopathological studies which emphasized the presence of intercellular fungal mycelium of DM pathogens into xylem of a variety of other hosts (Wehtje and Zimmer, 1978 and Fierro-Corrales *et al*., 2014). Current work indicated that the pathogen could not flow systematically upwards within the wood vessels. But, the fungal hyphae grew in the intercellular spaces of the parenchyma cells adjacent to xylem elements in the axis of the inflorescence as shown in Fig. (6 C).

It was found that oospores were hardly differentiated from the protoplasmic components and metabolic reserves into the cells of perisperm and embryonic tissues. Hence, application of the Grocott's methenamine silver stain (GMS) was very helpful as a staining procedure in tissue sections of the developing seed, where fungal structures were stained black (Fig. 7 B & C) by this method. The staining pattern of trypan blue, also revealed the occurrence of intercellular mycelium in the perisperm tissue (Fig. 7 D). Present study showed that plenty of oospores were located in the whole mounted perianth (Fig.7 A). This result is in consistence with findings of Danielsen *et al.* (2004) who reported that oospores of DM is located under the perianths of quinoa seed. Also, these results agree with those obtained by El-Assiuty *et al.* (2019) who reported that the structures of quinoa pathogen were present in all parts of quinoa seed. Moreover, findings we obtained throughout the present investigation have been confirmed by those found by Taha (2019) who proved by PCR-based method that the DM pathogen is present in all organ tissues at different growth stages of plant growth.

4. CONCLUSION

Results obtained throughout the present study showed definitely the presence of oospores of quinoa DM in pith of the radicle after 3 days of seed germination. Therefore, in relation to the dynamic of quinoa DM, treating quinoa seeds with an appropriate agent is a must to minimize the presence of oospores in the newly germinated seeds. In addition to the seed treatment, spraying plants at about 45 days of planting would be beneficial as complementary way of managing the disease since gametangia were strictly observed in the leaf mesophyll at this stage of plant growth.

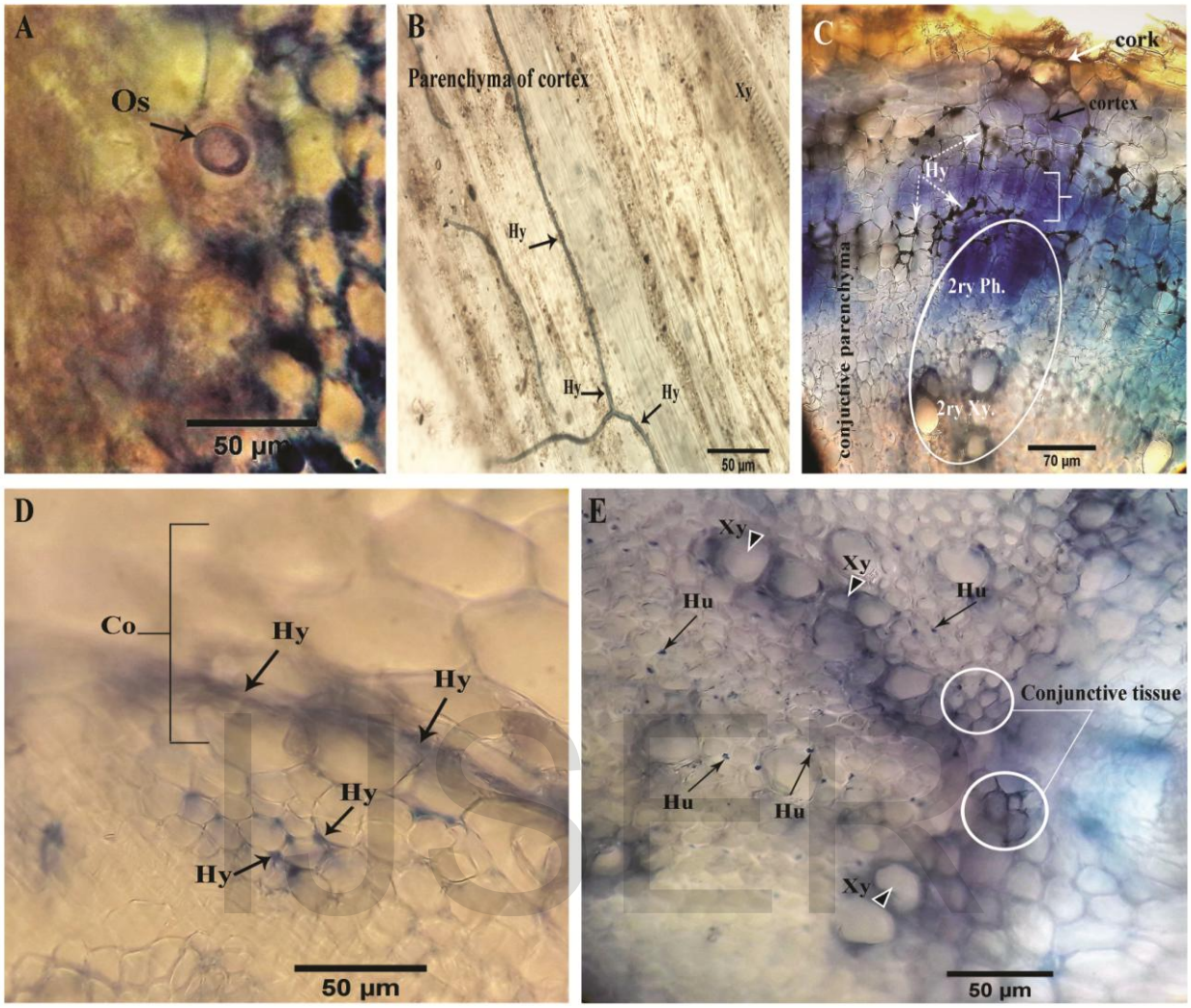

Fig.1. Fungal structures of *P.variabilis* detected in radicle and root tissues of quinoa plant

(A) Cross section in radicle after 3 days of seed germination, stained with trypan blue showing oospore within the pith region (arrow).

(B) Whole mount of radicle of 15-day old seedling, cleared in KOH and stained with trypan blue. Note the intercellular hyphae (Hy) among the parenchyma cells of the cortex. Xy is a portion of the vessel elements.

(C) Cross section of a trypan blue stained-tap root of 33-day old plant. Note the hyphae (Hy) in the intercellular spaces of differentiating secondary tissues (heads of white arrows and stippled shafts, delimited within the white brace), in the intercellular spaces of the inner layers of cortex (black arrow) and of the conjunctive tissue. Note the secondary vascular bundle (outlined with the oval template), consisted of secondary phloem and secondary xylem (2ry Ph and 2ry Xy).

(D) Transverse section of 80-day old plant root, showing blue-stained intercellular hyphae (Hy) in the region of conjunctive parenchyma (the two black arrows) of cortex (Co). Sections were cleared with chloral hydrate and lactic acid and stained with trypan blue.

(E) Cross section in root at harvest stage (120 days of planting) cleared according to Tisdall and Donnelly (1988) and stained with lactophenol-cotton blue. Note the intercellular mycelium in the parenchyma cells of the conjunctive tissue (outlined by the circular templates), and the haustorium (Hu) penetrating parenchyma cells (black arrows). The arrow heads show the vessel elements of the secondary xylem tissue (Xy).

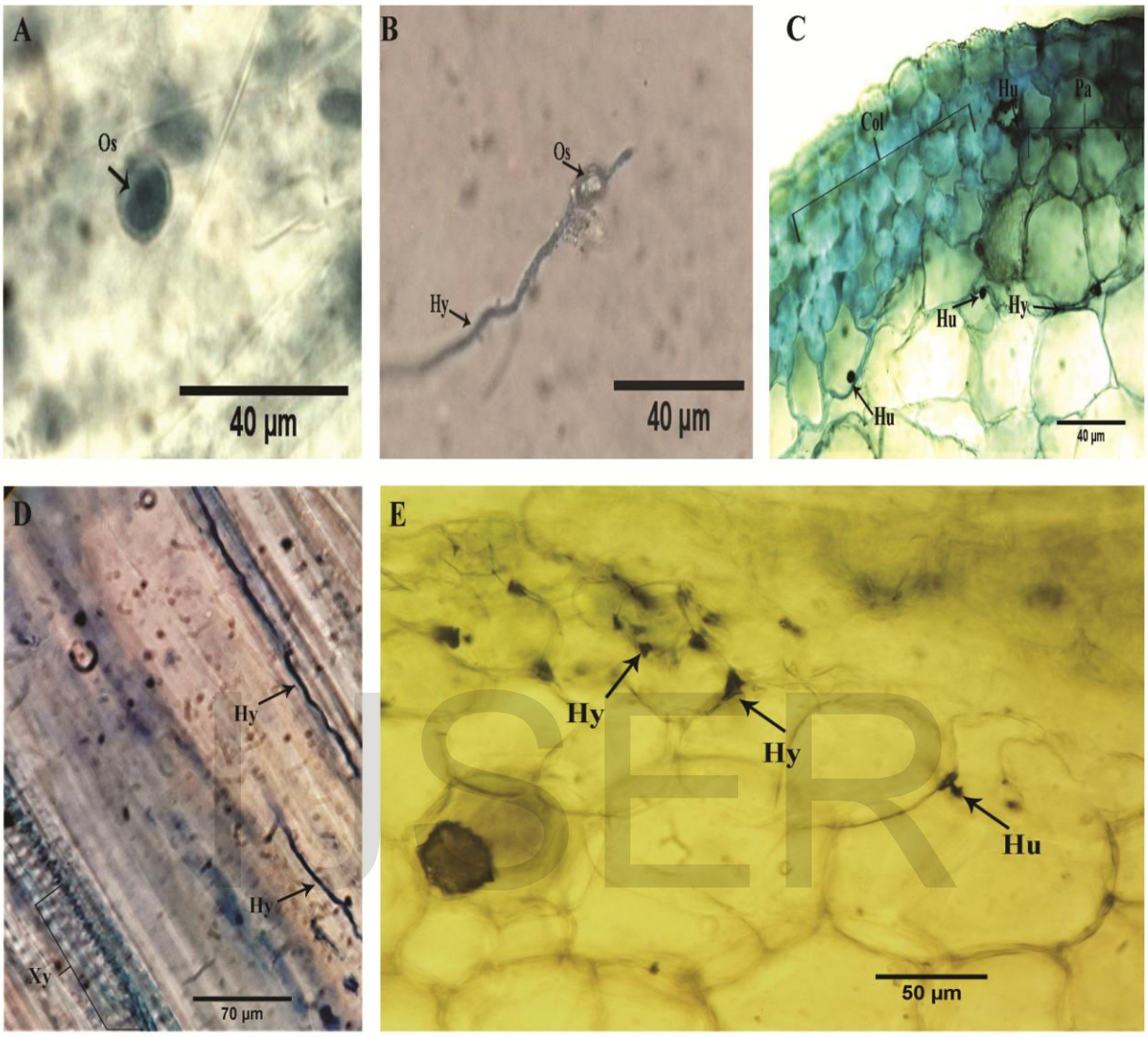

Fig.2. Fungal structures of *P.variabilis* detected in the hypocotyl and stem tissues of quinoa plants.

(A) & (B) Whole mounts of hypocotyls showing an un-germinated oospore (Os) after7days of seed germination (A) and germinated oospore (Os) in cortex tissues of 15-day old seedlings (B), note the two germ tubes of the oospore. The hypocotyles were cleared with KOH and stained with trypan blue.

(C) Cross section in stem of 60-day old plant cleared with KOH and stained with trypan blue. Note that the epidermis, followed by the cortex consists of two types of tissues; an outer collenchyma tissue of two or three cell layers followed by the parenchyma tissue has big cells with thin cellulosic walls. Note, also the hyphae (Hy) within the intercellular spaces of the collenchyma and the parenchyma cells of the cortex and the formation of button-like or spherical haustorium (Hu) in the cortical cells.

(D) Longitudinal section in stem of 80-day old plant cleared with KOH and stained with trypan blue. The stained-blue hyphae (Hy) are present in the intercellular spaces of parenchyma cells of the conjunctive tissue. Xy is a portion of the vessel elements of secondary xylem.

(E) Part of trypan blue stained-cross section of a stem of 120-day old plant, showing cortex cells have thin cellulosic walls, intercellular hyphae (Hy), and a haustorium (Hu) penetrating the thin cellulosic wall of a big parenchyma cell.

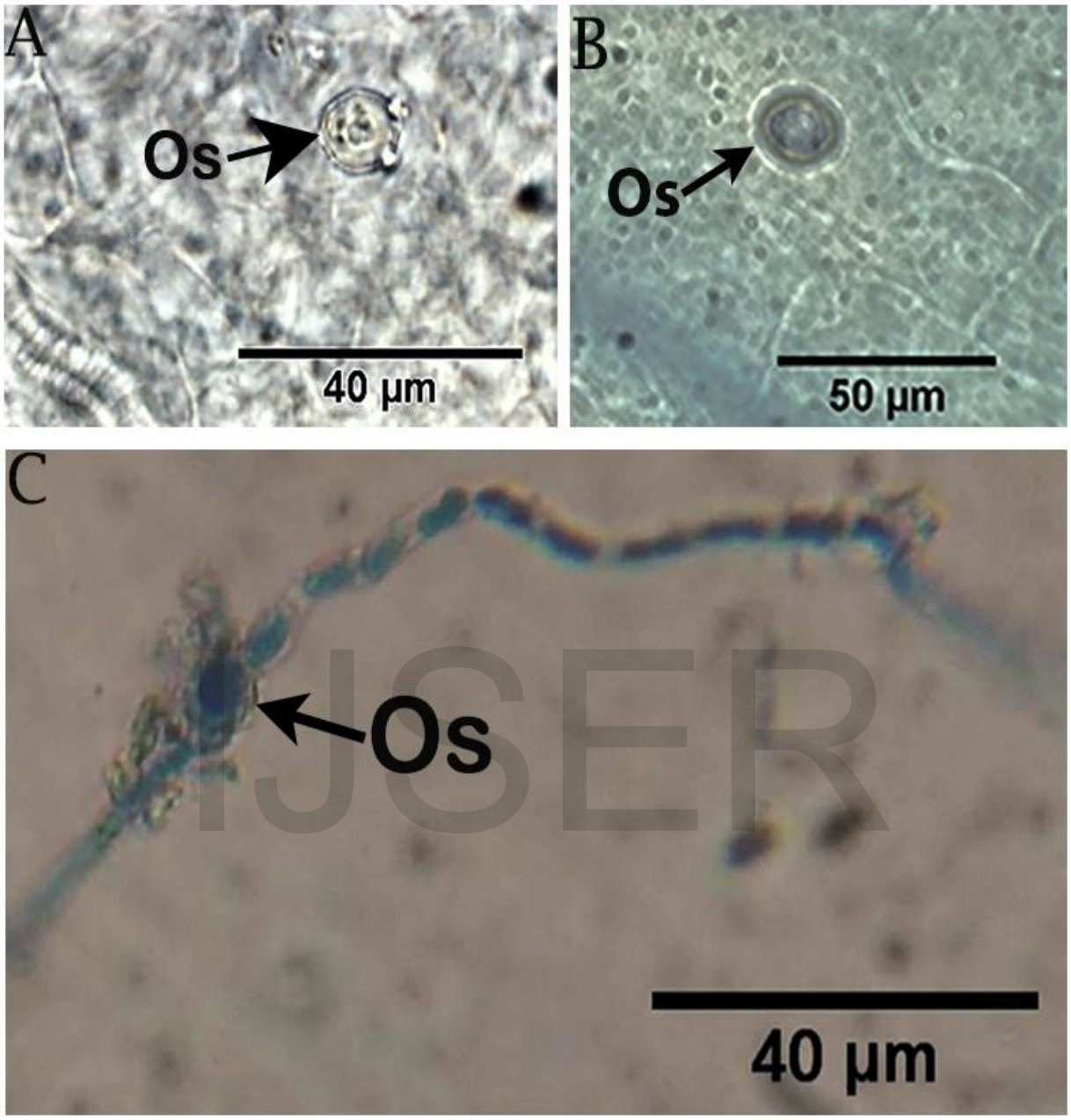

Fig.3. Oospores of *P. variabilis* early detected in the mesophyll tissues of cotyledon.

(A) Whole mount of cotyledon after 7 days of seed germination, cleared in KOH and stained with trypan blue showing an oospore (Os).

(B) Whole mount of cotyledon of 15-day old seedling, cleared in KOH and stained with trypan blue containing an oospore (Os). Note: The double wall and internal inclusions of oospore in (B), while it was thinner and lighter in (A).

(C) Whole mount of cotyledon of 15-day old seedling cleared in KOH and stained with trypan blue. Note the germinated oospore with two undulating form of two coenocytic germ tubes having fragmented protoplasm and dense inclusions

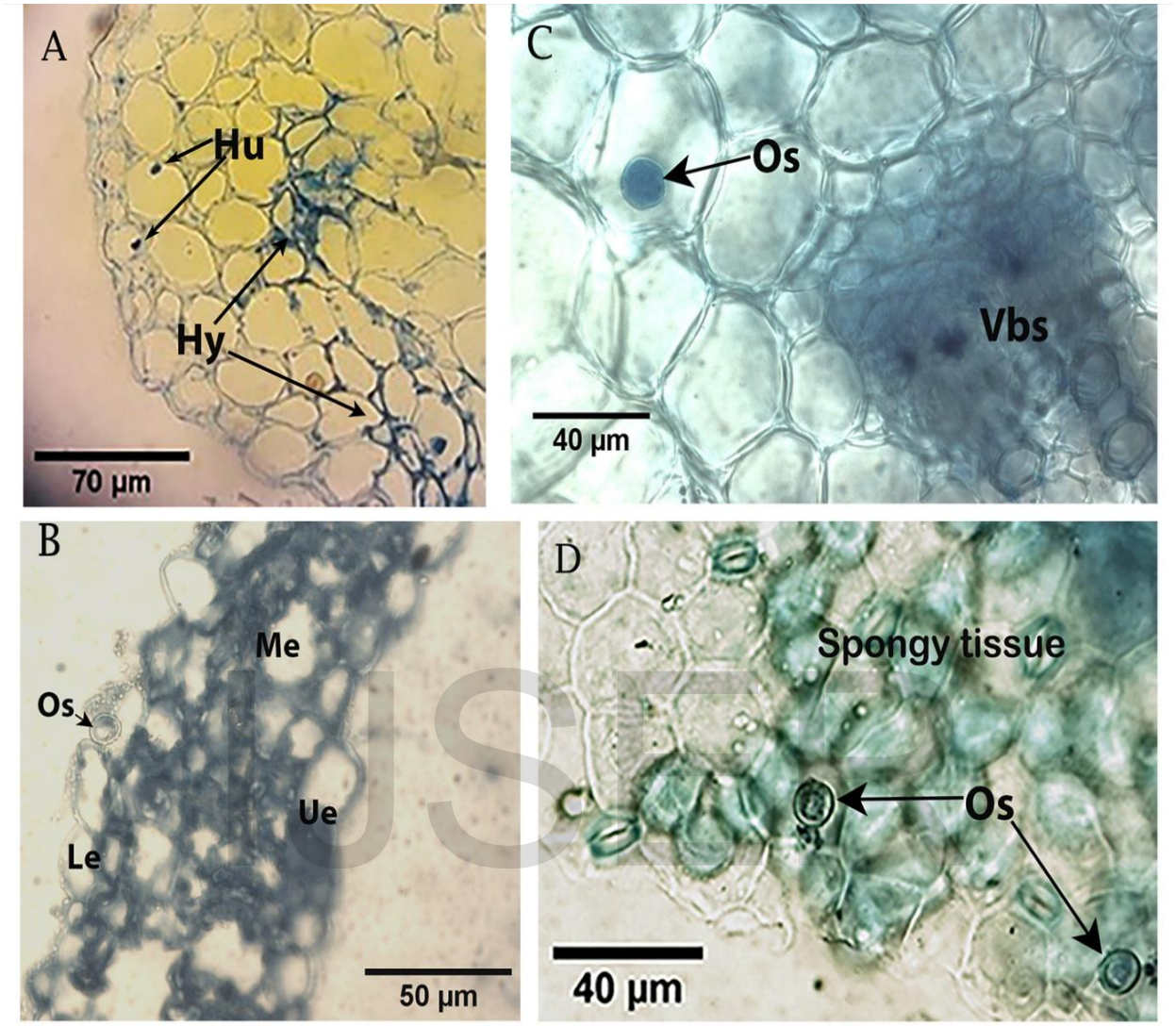

Fig.4. Fungal structures of *P. variabilis* detected in cross sections of leaves and petioles of 80 and 120-day old symptomatic plants .

(A) Cross section in the zone of leaf midrib of 80-day old plant stained with trypan blue. Note the growth of hyphae (Hy) within the intercellular spaces of the parenchyma cells and formation of button-like haustoria (Hu) on the parenchyma cells.

(B) Cross section in the leaf lamina of 80-day old plant stained with trypan blue, showing the upper epidermis (Ue), lower epidermis (Le) and the mesophyll tissue (Me) in between. Note disruption and collapse of the leaf lamina possible due to the displacement of the oospore (Os) from the spongy tissue to lower epidermis. Note, also the undifferentiated cytoplasm and the thin wall of the oospore at this stage of plant growth.

(C) Cross section in a leaf petiole of 120-day old plant stained with trypan blue, showing oospore (Os) with thick wall and dense inclusions located in the zone of parenchyma that surrounding the vascular bundles (Vbs).

(D) Paradermal section passing through the lower epidermis and the spongy tissue of the leaf lamina of 120-day old plant, cleared with KOH and stained with trypan blue. Note the occurrence of two oospores (Os) among the spongy tissue.

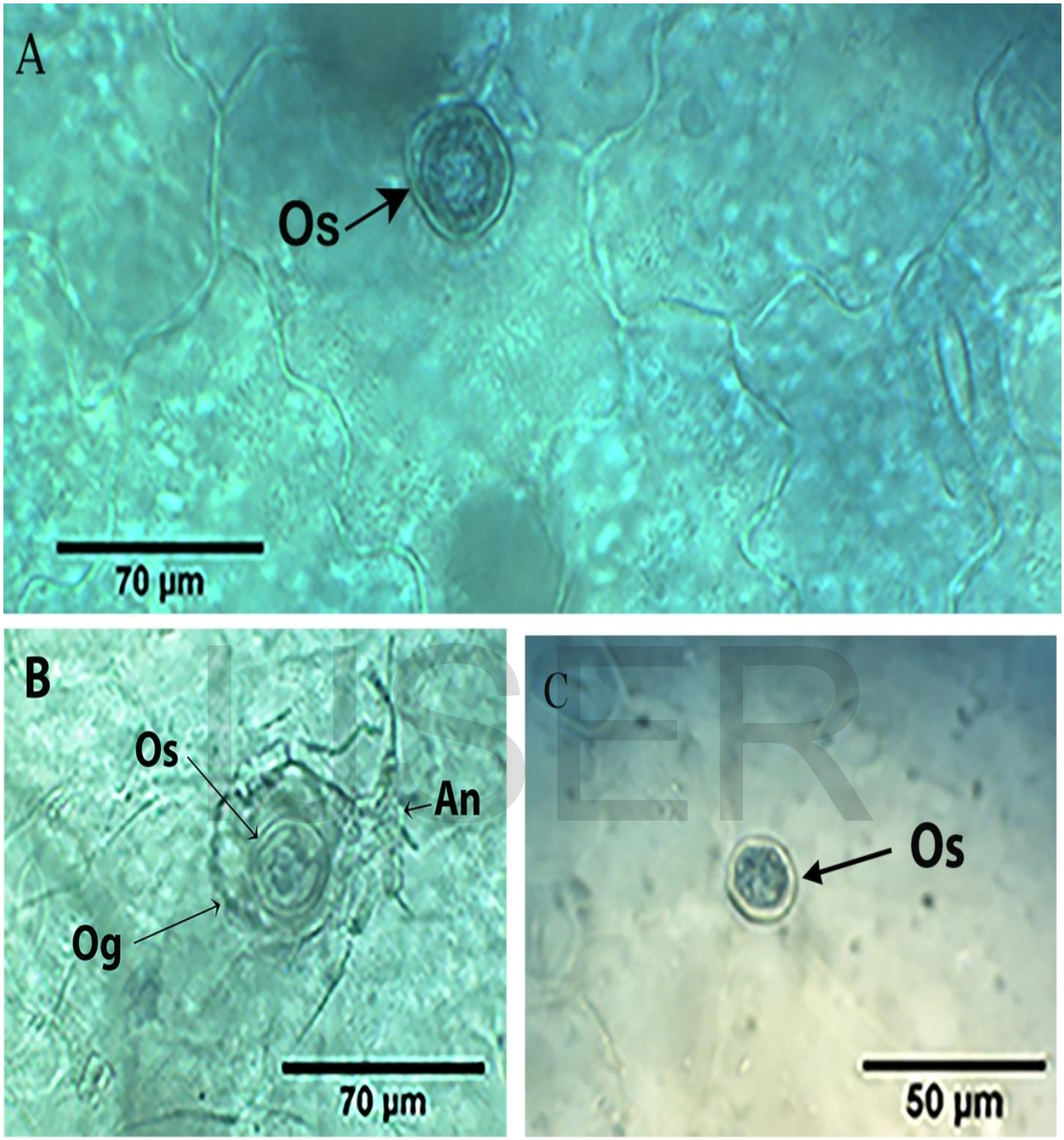

Fig.5. Oospores and gametangia of *P.variabilis* detected in whole mounts of leaves of symptomatic plant cleared with KOH and stained with trypan blue .

(A) Mature oospore (Os) with thick wall and dense protoplasmic inclusions in the leaf mesophyll tissue of 45-day old plant

(B) Oogonium (Og) attached to an antheridium (An) in leaf mesophyll tissue of 45-day old plant. Note that the developing oospore embedded into the oogonium has thin wall and less dense inclusions.

(C) Mature oospore (Os) with thick wall and dense inclusions present into the mesophyll tissue of 120-day old plat leaf.

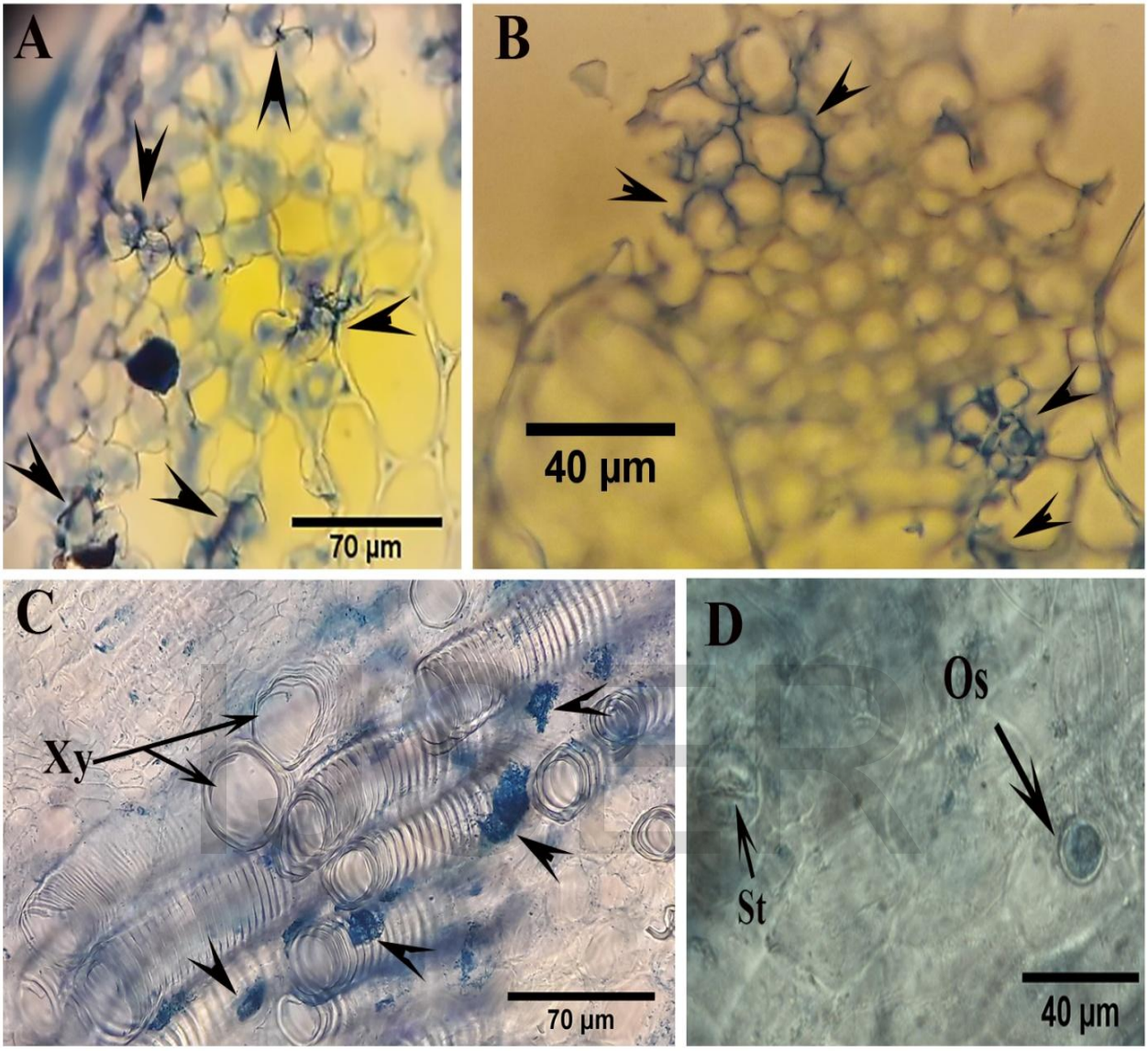

Fig.6. Fungal structures of *P. variabilis* detected in the principal axis of inflorescence of 80- day old symptomatic plant.

(A) Cross section in the principal axis stained with trypan blue. Note the bluish hyphae (black arrowheads) within the narrow intercellular spaces of the collenchyma cells of the cortex tissue.

(B) Cross section showing the presence of the fungal hyphae in the narrow intercellular spaces of the pith cells of the principal axis.

(C) An oblique cut (at angle between the cross and the longitudinal section) passing through xylem tissue of the axis, stained with trypan blue. Note: the xylem elements (Xy) have helical thickenings and simple perforation plates. The arrowheads show the dense fungal hyphae contiguous to the xylem elements.

(D) A strip of the outer epidermis and cortex tissues of the axis cleared with KOH and stained with trypan blue and inverted onto a glass slide to view the internal tissues. Note: the occurrence of a thick-walled mature oospore (Os), one stoma (St) and the epidermal cells with rounded or undulated walls.

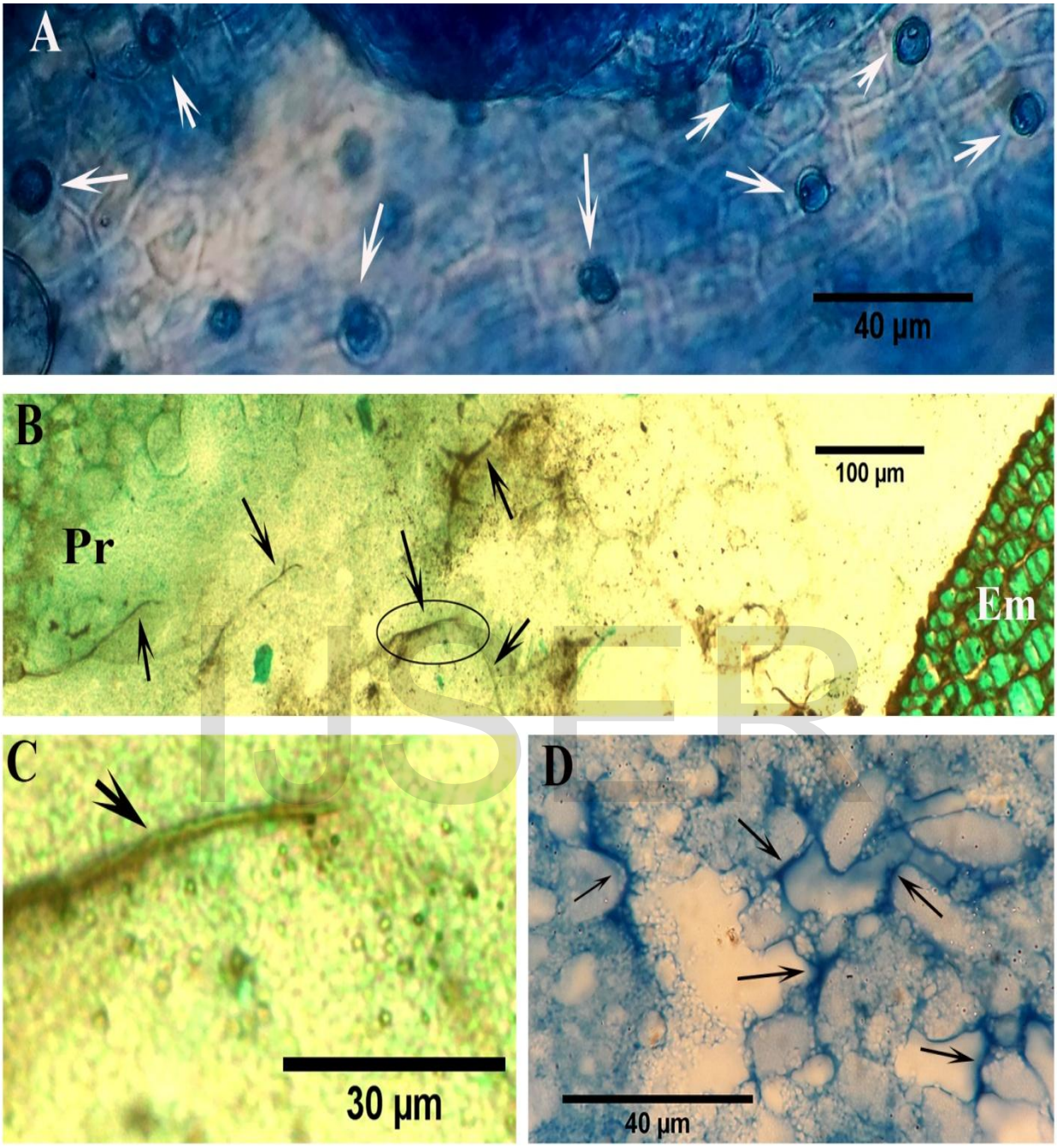

Fig.7. Fungal structures of *P.variabilis* detected in the perianth and perisperm of the developing seed of 80-day old systematic plants .

(A) Whole mount of the perianth cleared with KOH and stained with trypan blue showing plenty of thick-walled mature oospores (white arrows).

(B) Cross section in the developing quinoa seed stained with Methenamine silver nitrate viewing the black fungal hyphae (black arrows) in the perisperm tissue (Pr). Note: part of the developing embryo (Em).

(C) Magnifying portion of one coencytic hypha ahown in (B).

(D) Cross section in the perisperm tissue of the developing seed stained with trypan blue. Black arrows show the growth of hyphae (stained blue) within the narrow intercellular spaces of the perisperm.

Table 1. Presence of fungal structures of *P. variabilis* in different organ tissues of quinoa plant.

Organ	Age (days)	Tissue	Presence of Fungal Structures				
			Mycelium	Haustoria	Oogonia & Antheridia	Oospore (presence or absence and diameter (µm)	
						Developing (Immature)	Mature (thick walled and dense inclusions)
Radicle	3	Pith	-	-	-	-	+ 0.20
	15	Cortex	+	-	-	-	-
Root	33	Cortex	+	-	-	-	-
		Differentiating 2^{ry} tissues	+	-	-	-	-
		Conjunctive parenchyma	+	-	-	-	-
	80	Cortex	+	-	-	-	-
		Conjunctive parenchyma	+	-	-	-	-
	120	Conjunctive parenchyma	+	+	-	-	-
Hypocotyl	7	Cortex	-	-	-	-	+ 0.13
	15	Cortex	-	-	-	-	+ germinated
Cotyledon	7	In Whole mounts of mesophyll	-	-	-	-	+ 0.13
	15		-	-	-	-	+ 0.18
	15		-	-	-	-	+ germinated
Leaf lamina	45	Mesophyll	-	-	-	-	+ 0.30
		Mesophyll	-	-	+0.44 (oogonium)	+0.25	-
	80	Spongy tissue	-	-	-	-	+ 0.12
	120		-	-	-	-	+ 0.12
	120	Mesophyll	-	-	-	-	+ 0.17
Midrib	80	Parenchyma	+	+	-	-	-
Petiole	120	Parenchyma	-	-	-	-	+ 0.15
Axis of inflorescence	80	Collenchyma	+	-	-	-	-
		Pith	+	-	-	-	-
		Xylem Parenchyma	+	-	-	-	-
Developing seed perianth	80	Mesophyll	-	-	-	-	+ 13.4
Perisperm	80	Parenchyma	+	-	-	-	-

+ = Present, - = Absent.

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