ABSTRACT: 184

MACROPHOMINA PHASEOLINA: ATYPICAL PYCNIDIA PRESENCE AND SYMPTOMS IN SOYBEAN IN ARGENTINA

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Macrophomina phaseolina (Tassi) Goid is a soil-borne pathogen, infecting about 500 plant species. This fungus is an anamorphic Ascomycete and causes charcoal rot in soybean [*Glycine max* (L.) Merr.], one of the top ten important diseases affecting world production of the crop. Yield loss has been estimated to be up to 905 metric tones in 2006 in Argentina. Disease incidence has increased in recent years, especially when plants are under drought and high temperature stress. Microsclerotia formed on and inside the lower stem and roots are the diagnostic and more frequent signs. These structures permit long term survival in soil and serve as the primary source of inoculum. The pycnidial stage is only occasionally found in nature on soybeans. In March 2012, wilting and superficial lesions from above the soil line to the upper half of the main stem were observed in soybean plants from Villa Minetti in Santa Fe Province, Argentina. A reddish-brown discoloration was observed in the taproot vascular tissues, progressing upwards into the stem pith. *M. phaseolina* was isolated from excised pieces of symptomatic stem tissues as well as from pycnidia and microsclerotia. Dark, ostiolate pycnidia (160-179 µm), broadly ellipsoidal to obovoid, hyaline pycnidiospores ((14-)18-21(-25) × (6-)7-10(-11) µm) were observed, along with black variably-sized microsclerotia (50–150 µm), in accordance with what has been previously reported. The identity of isolates obtained from stem tissues, pycnidia and microsclerotia was confirmed by PCR using specific primers targeting an ITS region, which yielded an expected 350-bp amplified product. Atypical symptoms and signs pose the question as to whether there have been numerous careless disease reports from field soybeans which could have under-estimated the occurrence of charcoal rot. Additionally, pycnidia interspersed with microsclerotia on stem tissues could be easily confounded if not properly observed. Pycnidia and their conidia formed on field soybean plants would enable aerial dispersal, potentially changing the epidemiological aspects of this disease.

Macrophomina phaseolina: Atypical pycnidia presence and symptoms in soybean in Argentina

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RESEARCH CONFERENCE

THE PATHOGEN
Macrophomina phaseolina

Anamorphic Ascomycete - Family *Botryosphaeriaceae* (Crous *et al.*, 2006)

Soil inhabitant. Also seed-borne Widely distributed

More than 500 hosts reported

Primary source of inoculum → MICROSCLEROTIA. Long term survival in soil (more than 4 ys) under adverse conditions

PYCNIDIAL STAGE only occasionally in nature on soybeans (on certain hosts: aerial transmission)

Penetration → by roots, with mechanical pressure and enzymatic action, or through natural wounds. Invasion occurs 2 to 3 days after inoculation

(Watanabe, 1973; Dinghra, Sinclair, 1978; Ammon *et al*, 1975; Bressano *et al.*, 2010)

THE HOST: Soybean

Argentine: 3rd world producing country

Sown Hectares → 19,5 millions ha (2012/2013)

Production > 53 millons t (expected for current season)

Economic and social importance!

(Bolsa de Comercio de Rosario, 24/1/2013)

THE DISEASE: CHARCOAL ROT OF SOYBEAN (CRS)

Plants susceptible at any stage

Early infections may stay latent → when proper conditions occurs…

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Argentine: 3rd world producing country

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Production > 53 millons t (expected for current season)

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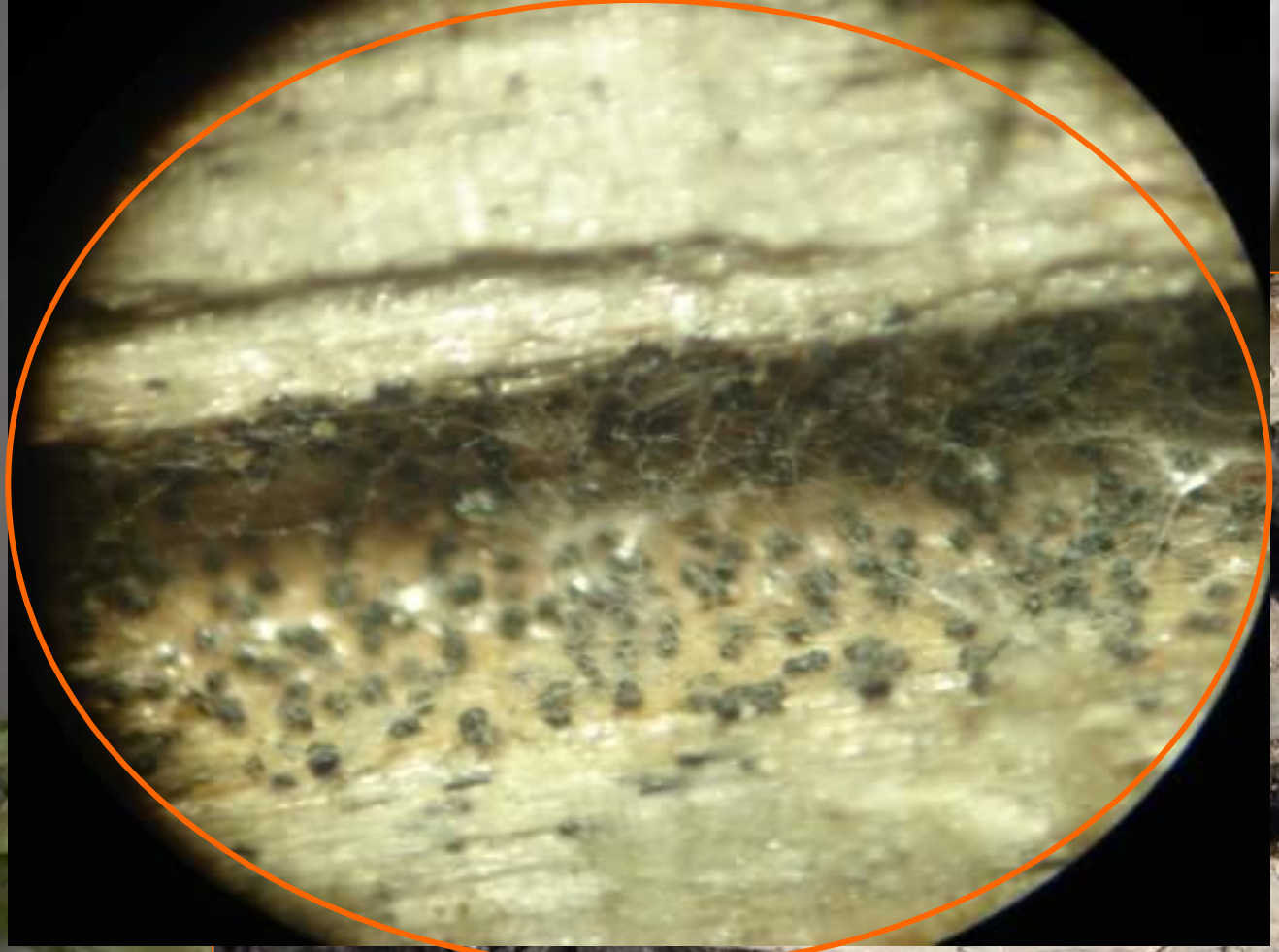
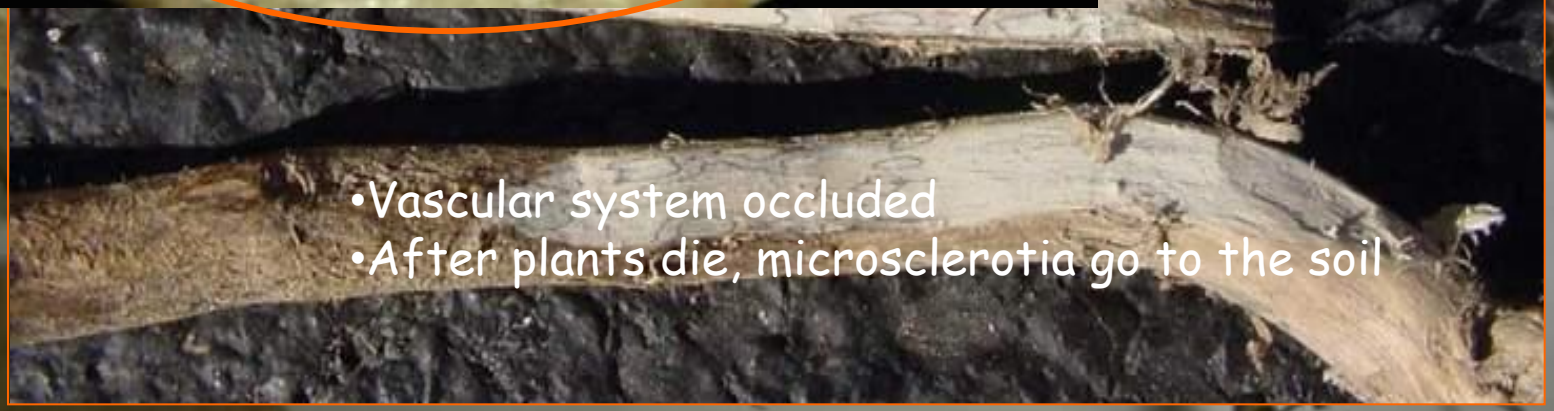
THE DISEASE: CHARCOAL ROT OF SOYBEAN

SYMPTOMS

- Wilted and/or dead plants
- Dry leaves stay attached to the stem
- Affected plants in spots on the field
- Black to gray or brown discoloration of the stem tissues
- Vascular system occluded
- After plants die, microsclerotia back to the soil





•Vascular system occluded
•After plants die, microsclerotia go to the soil

•Black to gray or brown discoloration of the stem tissues

- **THE OPTIMAL ENVIRONMENT**

Air and soil temperatures → 28 to 35 C
Limited soil moisture → -0,08 to – 2,0 MPa
Tolerance to drought conditions →competitive advantage

(Sinclair and Backman, 1989; Cervantes-García *et al.*, 2003)

- **AGRONOMIC IMPACT of CRS**

CRS → top ten most important disease affecting world soybean production.

Afects root volume and weight, height, and amount and size of leaves → **yield losses**

Scarce data! (In Brasil → 50% losses; in USA → 6 % to 33% under irrigation! In Argentina → losses up to 100%, in Santa Fe)

(Sinclair and Gray, 1972; Ferreira *et al.*, 1979; Atlas fitopatológico, 2011)

The current case…

March 2012, Laboratory of Plant Pathology of the Balcarce Integrated Unit, at the INTA Experimental Station, Buenos Aires, Argentina.

Soybean plants (cultivar A8000 RG) from Villa Minetti in Santa Fe Province, Argentina.

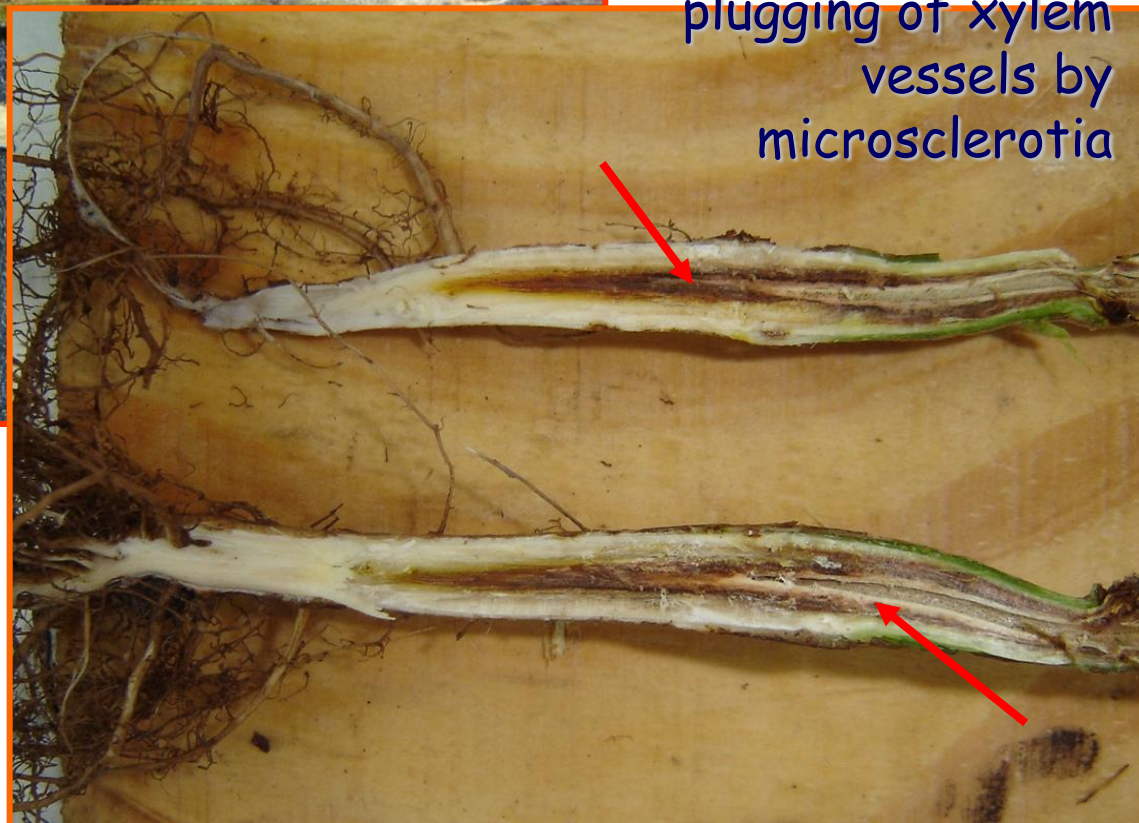
Plants with different symptoms and signs:

- wilting and superficial lesions from soil line to upper half of main stem
- Reddish brown discoloration in taproot vascular tissues, progressing upwards into stem pith
- On stems lesions: pycnidia and microsclerotia
- Grey discolouration of infected stems caused by pepper-like microsclerotia

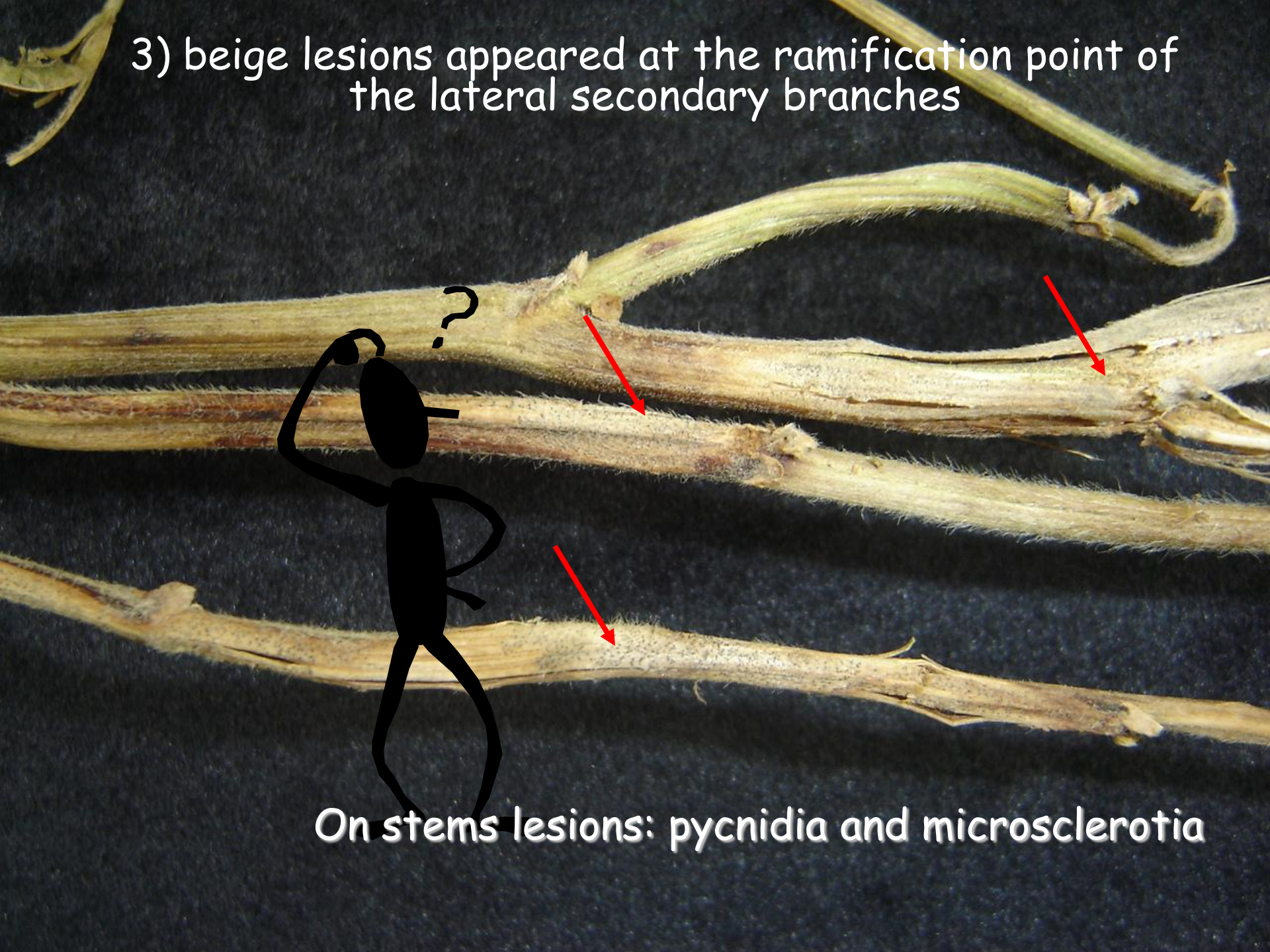
1) wilting, drying of the whole plant and superficial lesions from soil line to upper half of main stem

2) circular to oblong, reddish-brown, superficial lesions on the stem, showing a canker-like appearance

This symptom was accompanied by discoloration of the vascular elements of roots and lower stem, necrosis of the roots, stems and mechanical plugging of xylem vessels by microsclerotia

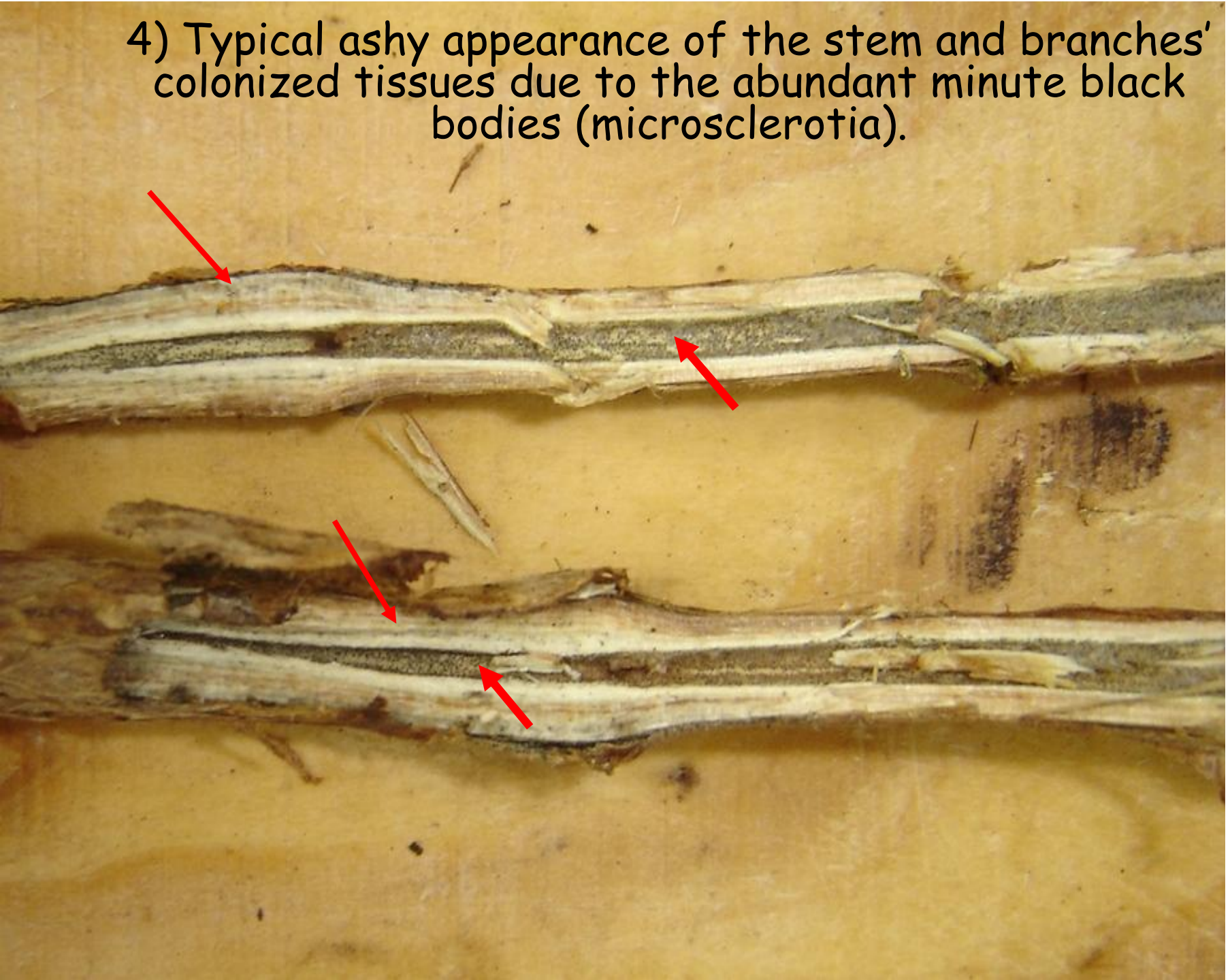


3) beige lesions appeared at the ramification point of the lateral secondary branches



On stems lesions: pycnidia and microsclerotia

4) Typical ashy appearance of the stem and branches' colonized tissues due to the abundant minute black bodies (microsclerotia).

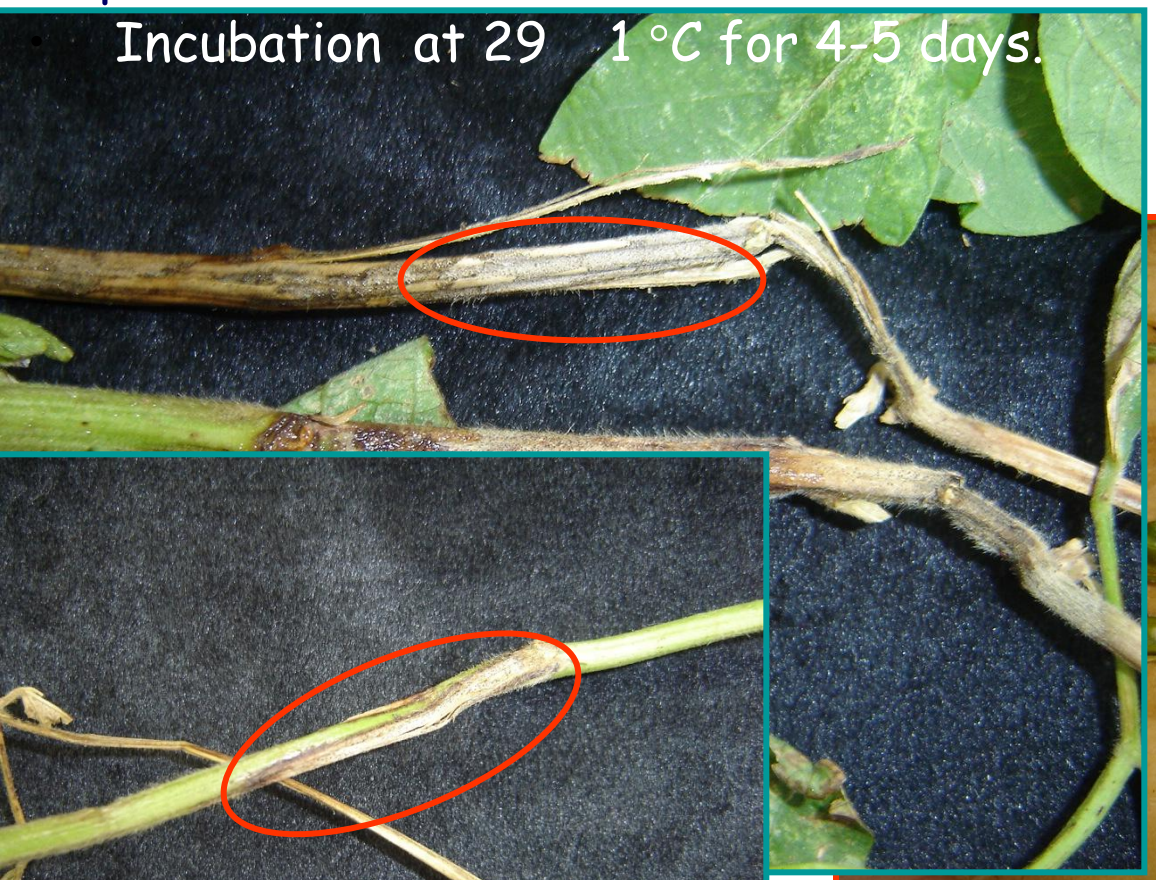


OBJECTIVE

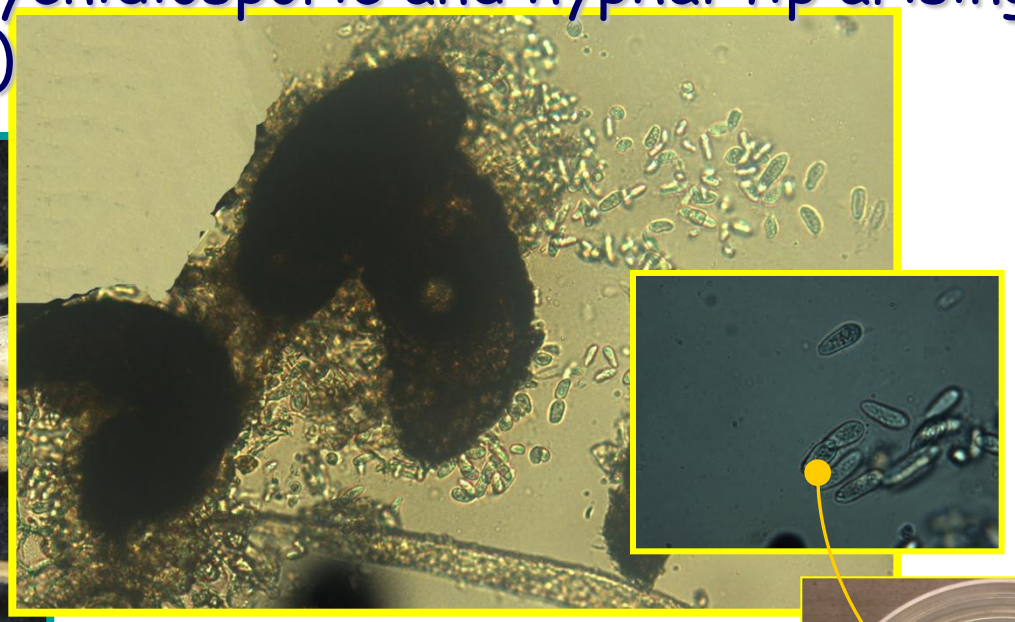
To determine whether *Macrophomina phaseolina* is the causal agent with atypical symptoms and sign on soybean stems.

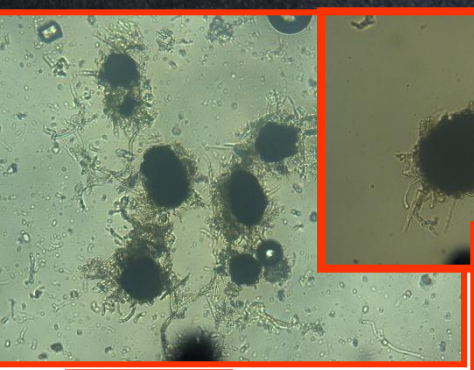
MATERIALS AND METHODS

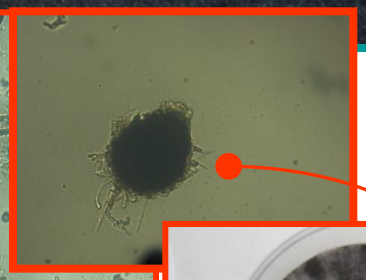
- Stems with lesions incubated in moist chambers, conditions of the laboratory.
- Isolation: 2–3 mm-long pieces of symptomatic stems disinfected and plated on Petri dishes with 2 % APDA
- Incubation at 29 1 °C for 4-5 days.



1. Pure cultures (monopycnidiosporic and hyphal tip arising from one microcletotia)

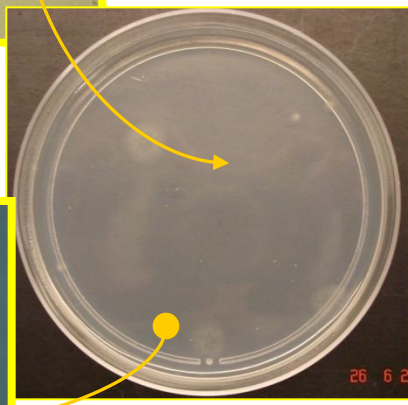






Mp

?



?

APDA, incubated at
29 1 °C for 4-5 days.

MATERIALS AND METHODS

2. Measurement of pycnidiospores *in vivo*

Length and width of 30 pycnidiospores from each of 5 pycnidia were measured

Photographs (400x) of pycnidia and their conidia were taken. Olympus BX61 microscope with a specific software (Image-Pro AMS 6.1-Media Cybernetics Inc., USA) was used to measure.

MATERIALS AND METHODS

To confirm the identity of the isolates of *M. phaseolina* (Mp1 and Mp2)

3. Molecular identification

- Grown and maintained on APDA for 3 days at 29 1 °C. Fungal mycelia for DNA extraction were harvested.
- Total genomic DNA was extracted using a NucleoSpin ® Plant II Kit (MACHEREY-NAGEL).
- Negative controls: *Verticillium dahliae* (Vd) and *Fusarium verticillioides* (Fv)
- Positive control *M. phaseolina:* isolate from Juan N. Fernandez, Buenos Aires Province (JNF)..
- PCR amplifications using specific primers MpKFI (forward) and MpKRI (reverse) (Kishore Babu *et al.*, 2007)


Bolivia
Paraguay
Brasil
Uruguay
Chile
Océano Pacífico Sur
Océano Atlántico Sur
a
b
a
b
Is. Malvinas
(Arg.)
Antártida
Argentina

MATERIALS AND METHODS

4. Pathogenicity test

- Pathogenicity testing was carried out at the seedling stage - Manici *et al* (1995) method, with modifications (i.e. a sterile soil layer between fungal colony and soybean seeds).
- 3 isolates: Mp1 from pycnidispore; Mp2 stem pith lesion, both Villa Minetti, Santa Fe and JNF stem pith from Juan N Fernández, Buenos Aires Province).
- Once petri dishes were completely covered by the fungal growth, a 0,5 cm-thick sterile soil layer were placed above the colony in order to separate the seeds from direct – otherwise too aggressive-contact with the mycelium.
- Commercial cvs tested: A8000 RG, DM7.0 RR and SPS3900 RR
- Seeds were sterilized with 2 % sodium hypochlorite for 5 min and rinsed twice in sterile tap water.
- 6 seeds/petri dish/from each soybean genotype were placed on the soil layer of the different isolates.
- 10 petri dishes were sealed and incubated at 29 1 °C in darkness. Pathogenicity was recorded 7 days after inoculation as and the damage severity caused by the fungus to the seed and seedlings. Also the proportion of germinated seeds was recorded.

Scale described by Manici *et al.* (1995)

0= healthy seed,
1 = discoloration of a portion of the seed in contact with the mycelium,
2 = seed teguments invaded by mycelium and microsclerotia, but healthy seedling,
3 = seed teguments free from the fungus, but seedling infected by the pathogen,
4= both teguments and seeds infected,
5 = seed invaded by the fungus and not germinated.

✓Seeds from each cv were sown in 3 replications in a completely randomized design.

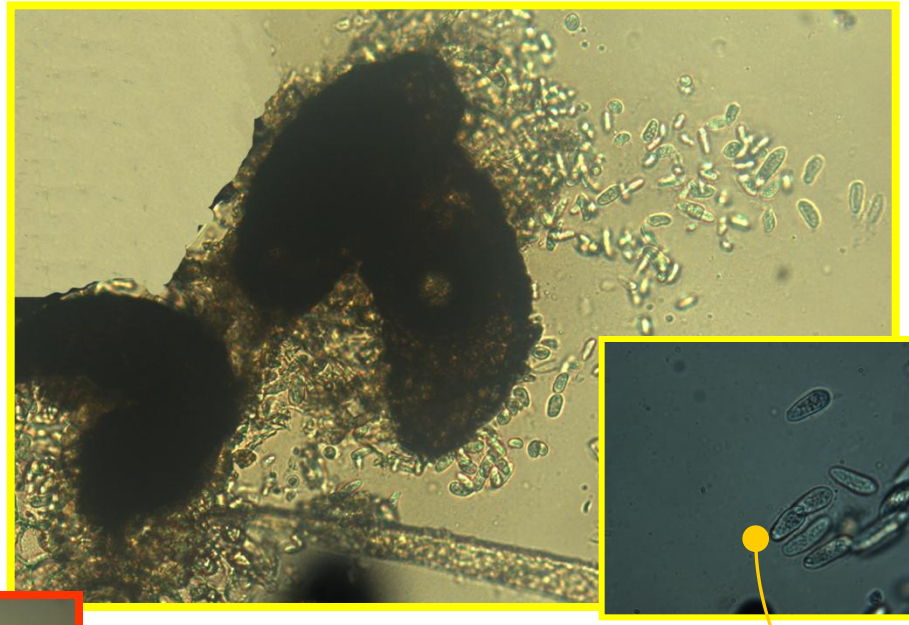
✓Variance of data was analyzed and isolate effect means on every soybean genotype were compared using Duncan test ($\alpha < 0.05$) InfoStat 2.0. (2008, Córdoba, Argentina) .

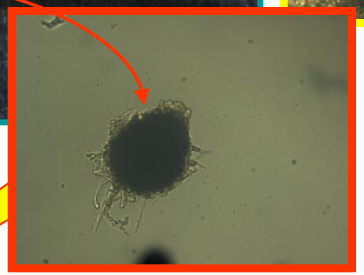
RESULTS

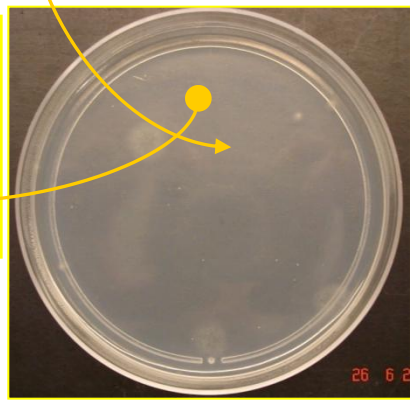
- Colonies with abundant microscelerotia developed from symptomatic tissues as well as from pycnidia when cultured on APDA giving the typical aspect of grayish to black colony of *M. phaseolina*.
- Black, variably-sized (50-150 µm) microsclerotia were observed.
- A consistent difference was also observed among the type of growth and aspect of the colonies from stem canker-like lesion (Mp1) and pycnidiopore (Mp2) isolates.

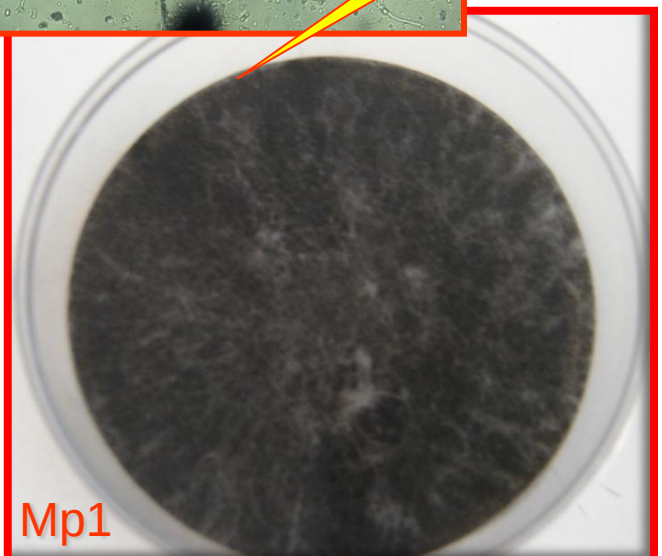
- Colony type and color, and sizes of the rest of the fungal structures were all similar to those previously published.

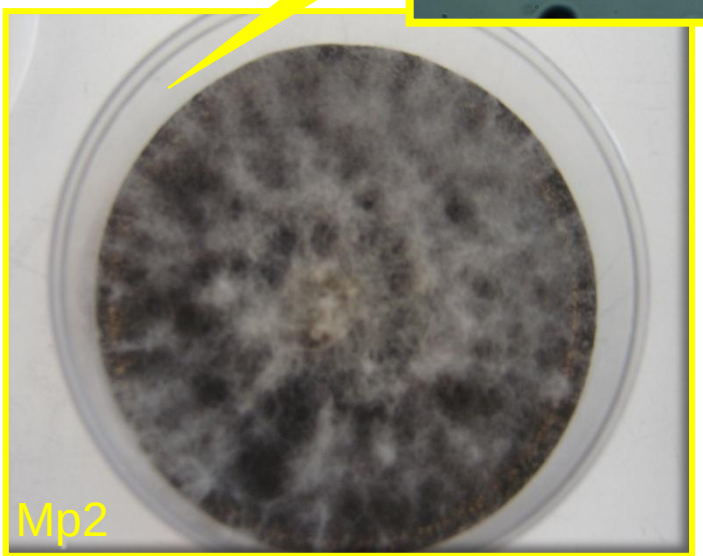
1. Pure cultures











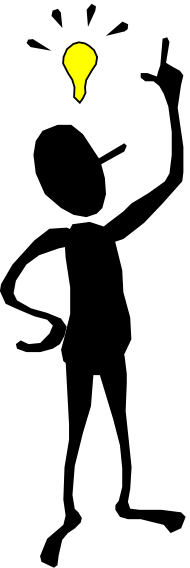
RESULTS

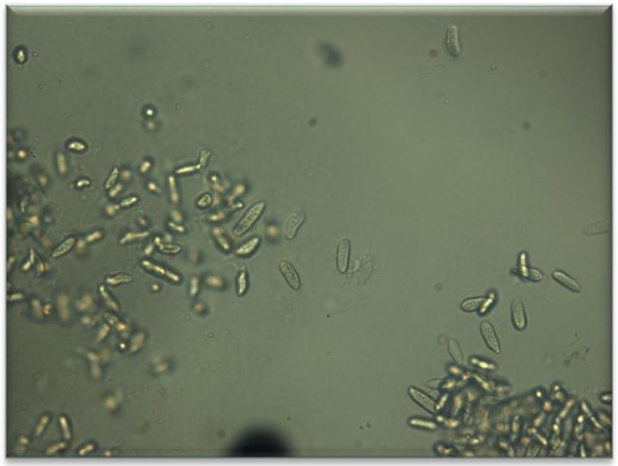
1. Pure cultures

The fungus was isolated and positively identificated as *M. phaseolina* by traditional examination and culturing from the different lesion types and signs found on soybean plants in the sample.

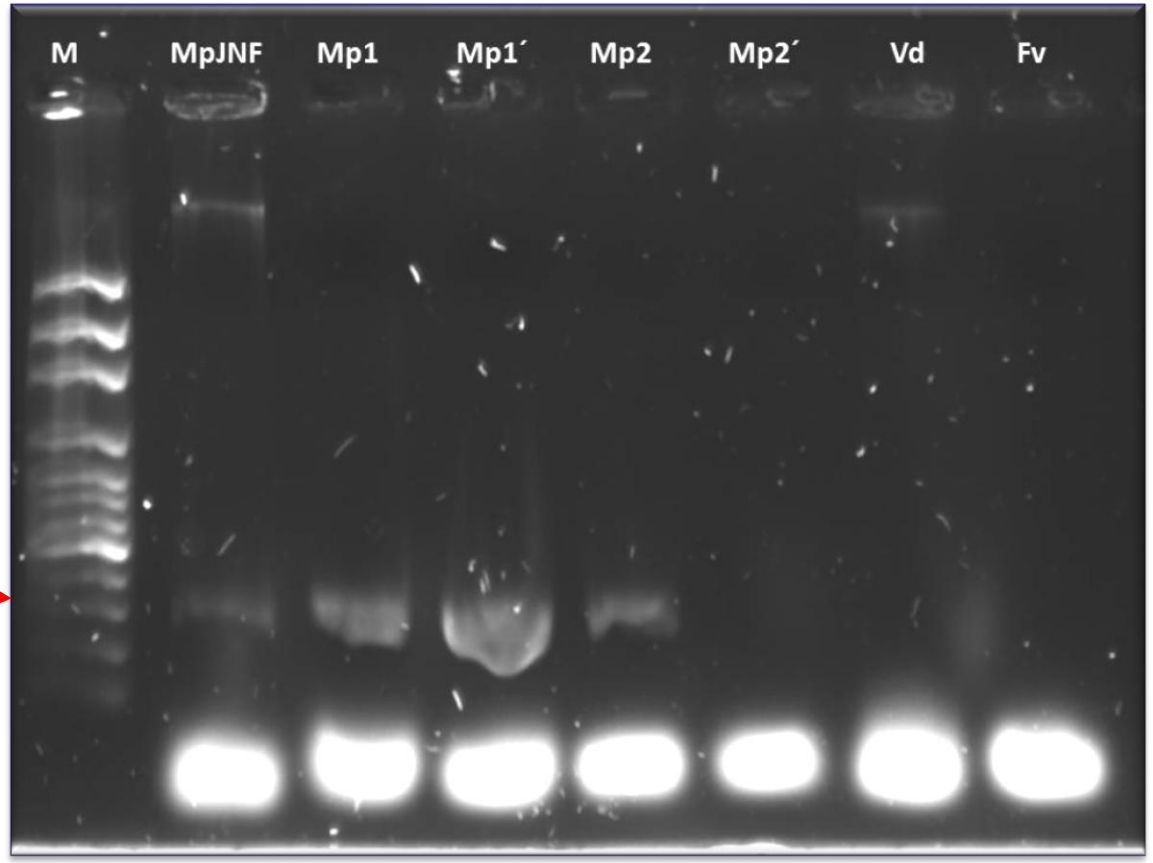
2. Medition pycnidiospores *in vivo*

Unicellular, elliptical to ovoid pycnidiospores (16-)20-24(-32) x (6-)7-9(-11) µm.





3. Molecular idenfication



Band patterns from genomic DNA amplified by PCR using specific primers for *Macrophomina phaseolina (*MpKFI-forward and MpKRI-reverse, Kishore Babu *et al.*, 2007): from left to right, size marker (M); stem canker-like lesion and pycnidiosporic isolates from Villa Minetti (Mp1 and Mp2); *Verticillium dahliae* (Vd), *Fusarium verticillioides* (Fv) (both negative controls); and *M. phaseolina* isolate from Juan N. Fernandez, Buenos Aires Province (MpJNF, positive control).

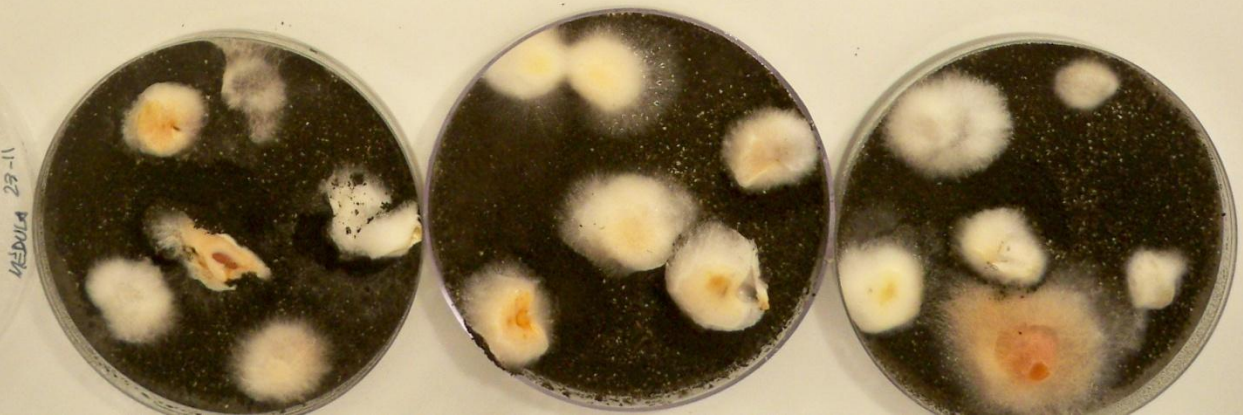
A8000 cultivar

4. PATHOGENICITY TEST

BUENOS AIRES,
JNF isolate →
(less pathogenic, more germinated seeds)

SANTA FE
Mp2 Pycnidiospore isolate →
(Mainly microsclerotia on seed teguments)

SANTA FE,
Mp1 Stem canker-like isolate →
(Mainly abundant fluffy mycelium on seeds)

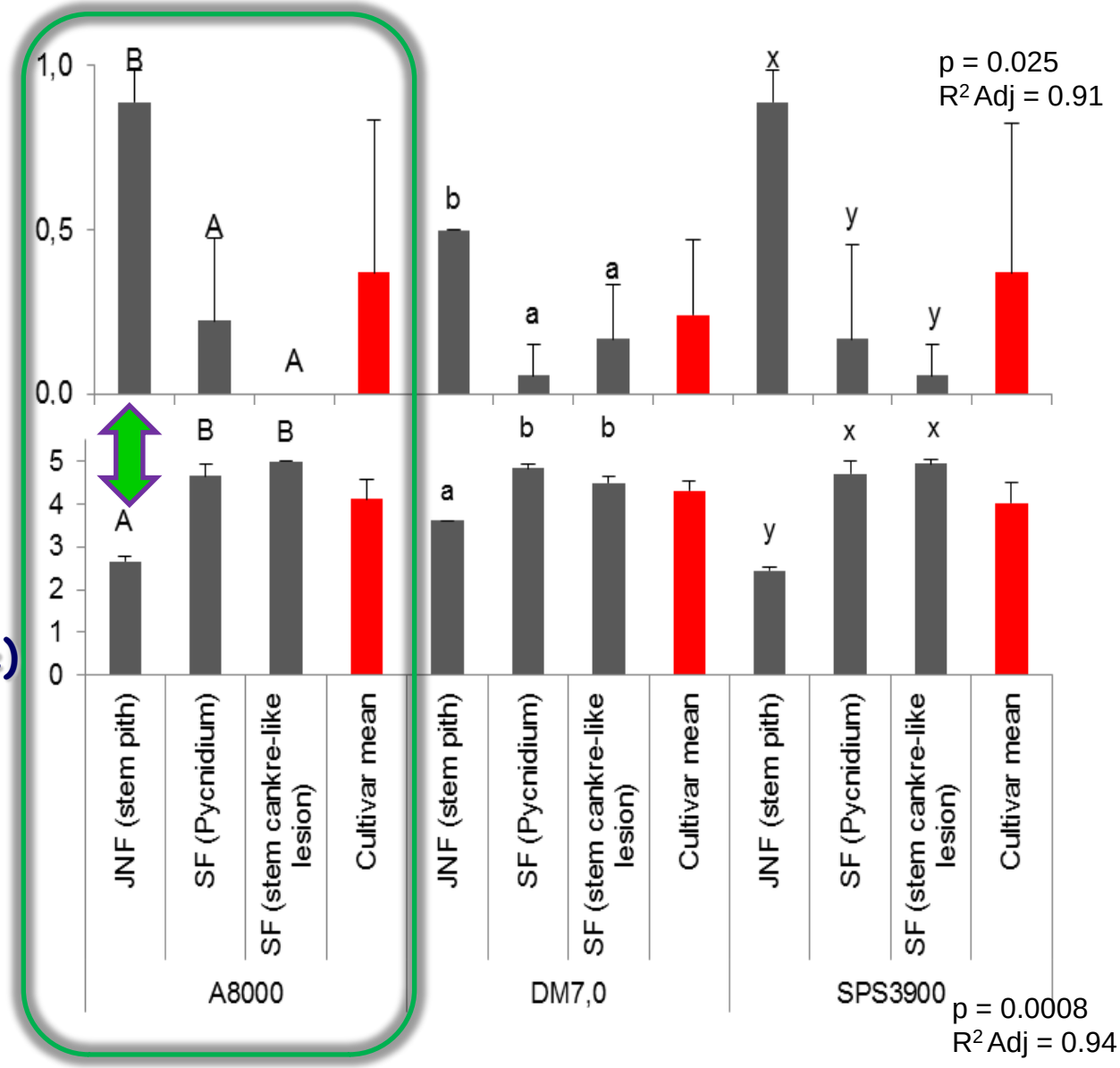


4. PATHOGENICITY TEST

Seed germination (expressed as proportion)

Similar behavior in the other soybean cultivars

Damage severity (0 to 5 scale)



CONCLUSIONS

- ✓ *Macrophomina phaseolina* was confirmed as the fungus responsible for symptoms and sign observed in the plants sampled.

- ✓ Atypical symptoms and sign pose the question as to whether there have been numerous careless disease reports from field soybeans which have underestimated the occurrence of charcoal rot.

- ✓ Pycnidia interspersed with microsclerotia on stems could be easily **confounded** if not properly observed.

    - ✓ *Would pycnidia and their spores formed on field soybean plants enable aerial dispersal, potentially changing the epidemiological aspects of this disease?*


¡Muchas Gracias!
¡Thank You Very Much!