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Freckles, a blemish of onion bulbs caused by *Sclerotium* sp.

Peca, un defecto menor en bulbos de cebolla causado por *Sclerotium* sp.

Kiehr M & R Delhey

Abstract. Freckles is a new blemish of white-scaled onion bulbs in the Lower Rio Colorado Valley (Argentina) characterized by the formation of small sclerotia imbedded in the dry scale tissue. The causal agent has been isolated from scales and symptomless roots of onion and was identified as *Sclerotium* sp. Although in its host range and morphology it resembles *Sclerotium cepivorum*, it is quite different with respect to other cultural and pathological traits. In inoculation experiments, onion and garlic plants could be infected and the typical sclerotia were formed, but no rot, a symptom characteristic for *S. cepivorum*, nor any other tissue deterioration was observed. The inoculated fungus could be re-isolated fulfilling Koch´s postulates. Freckles is considered a minor imperfection of minimal, if any, economic importance.

Keywords: *Sclerotium* sp.; *Sclerotium cepivorum*; Onion; Argentina.

Resumen. Peca es un cuadro sintomático nuevo que fue detectado en bulbos de cebolla de túnica blanca, en el Valle Bonaerense del Río Colorado, Argentina. Se caracteriza por la presencia de diminutos esclerocios oscuros, incrustados en las catáfilas externas secas. Se aisló al agente causal a partir de catáfilas afectadas y de raíces asintomáticas de cebolla y se lo identificó como *Sclerotium* sp. En cuanto a ciertas características morfológicas y al rango de hospedantes es similar a *Sclerotium cepivorum*. Sin embargo, difiere de esta última especie en cuanto al comportamiento en medio de cultivo y a sus características patológicas. Bulbos y plántulas de cebolla y bulbillos de ajo inoculados experimentalmente con *Sclerotium* sp. desarrollaron los típicos esclerocios; se reaisló el hongo inoculado dando cumplimiento a los postulados de Koch. Nunca se observó podredumbre ni otro deterioro o muerte de tejido, síntomas típicos de *S. cepivorum*. Se considera que peca es un defecto menor de escasa o nula importancia económica para el cultivo de cebolla.

Palabras clave: *Sclerotium* sp.; *Sclerotium cepivorum*; Cebolla; Argentina.

Departamento de Agronomía, Universidad Nacional del Sur, 8000 Bahía Blanca, Argentina.
Address Correspondence to: Dr. Mirta Kiehr, *e-mail*: mkiehr@criba.edu.ar
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INTRODUCTION

The Lower Rio Colorado Valley (LRCV; 39-40° S; 62-63° W) is the main onion growing area in Argentina; diseases are an important factor which limit yields and affect commercialization (Kiehr et al., 1996). Since the early 1990s, a hitherto unknown symptom called our attention, "freckles", which appeared sporadically on white-scaled onion bulbs during storage. Freckles owes its name to the small dark-brown to black dots which appear in the dry outer bulb scales, mainly on their basal portion. When studied microscopically such dots turned out to be sclerotia imbedded in the tissue. Here we describe the causal agent and we present the results of inoculation tests.

MATERIALS AND METHODS

To isolate the causal agent, sclerotia-bearing bulb scales were surface sterilized (NaOCl 2%, 1 min), cut with a scalpel in small pieces which were transferred to Petri dishes with potato dextrose agar (PDA). Cultural, morphological and morphometric studies were carried out in PDA with two of the isolates obtained: SnS from the bulb of an unknown white-scaled cultivar and SnO obtained during routine isolations from symptomless roots of a field-grown plant of onion cv. Valcatorce. These isolates of the unknown fungus were compared with two isolates of *Sclerotium cepivorum* obtained from onion with typical white rot symptoms: ScA (Neustadt a. d. Weinstr., Germany) and ScC (Bahía Blanca, Argentina).

The *in vitro* growth rate was determined in 9 cm Petri dishes with PDA. Five-mm plugs of actively growing colonies of isolates SnO, SnS and ScC were placed in the centre of each dish and the colony diameter was measured daily taking two diameters in right angle until all the colonies had reached the edge of the dish. The cultures were incubated at room temperature (20-23 °C) in the dark. There were three replicates. A similar test was carried out with isolates SnO, SnS and ScA, with two replicates.

The mycelial compatibility tests were carried out in similar conditions, following the procedure of Earnshaw and Boland (1997), but without the addition of "red food colouring" to the PDA medium. Plugs cut from fungal colonies were placed in dishes at a distance of 4 cm. Self-self and self-nonself pairings of the four isolates were carried out, with four replicates for each pairing.

For the pathogenicity tests, three-week-old colonies of isolates SnO and SnS growing in PDA were incorporated in sterile soil; for the control, only PDA was incorporated. Healthy-looking medium-sized bulbs of the yellow-scaled cv. Valcatorce and the white-scaled cv. Gladstone were cleaned from adhering soil, rests of roots and loose scales, submerged in 96% ethanol, flamed shortly in the region of the disk and planted in the pots with the soil-inoculum mixture. Four pots of each treatment were grown in the laboratory at approximately 20 to 23 °C and diffuse light and the other four in a growth chamber at 27 ± 2 °C and artificial light. After four weeks, the plants were harvested and examined for symptoms (Experiment 1).

Onion seeds cv. Valcatorce were externally sterilized (NaOCl 2%, 1 min) and germinated between moist filter paper. When approximately 2 cm long, the seedlings were transplanted to soil infested with isolate SnS or to uninfested soil (control), 20 seedlings/plastic tray and six trays/treatment. The trays were distributed randomly in a growth room at 25 to 30 °C and artificial light. After two months, plant survival, plant height and root length were determined and the data were analyzed by ANOVA (Experiment 2).

In a similar way, externally sterilized bulblets of garlic cv. Blanco were planted in plastic pots filled with soil infested with SnO and SnS and in uninfested control soil. Six pots per treatment, with two bulblets each, were incubated in the same growth room as before during two months (Experiment 3).

RESULTS

Fungal morphology. The sclerotia are arranged more or less uniformly, mainly on the lower part of the outer bulb scales (Fig. 1 A, B, D). They are totally embedded in the scale tissue but small protuberances may stick out on the overlying epidermis. Sclerotia begin to form as very small hyaline hyphal conglomerates which increase in size and darken. Mature sclerotia are more or less globose when growing in agar culture or on the roots and somewhat flattened in the scales. They have a black rugose surface and measure 0.2 - 0.4 mm in diameter, but they may reach up to 0.6 mm and have a more irregular shape when two sclerotium initials coalesce. There is a thin outer rind that consists of a few layers of thick-walled melanized cells and a medulla of hyaline elongate prosenchymatic cells (Fig. 1 C).

The hyphae formed on PDA are hyaline, 2.5 to 13 µm in diameter, and branch in acute angles, with septa formed near the branching point. No clamp connections were found. The aerial mycelium formed on the agar plate is white, turning later into light brown (Fig. 3 A). It is very scarcely developed in SnO and slightly more so in SnS; the aerial mycelium is more abundant in the two *S. cepivorum* isolates grown in the same conditions. When all the agar surface is colonized numerous uniformly distributed sclerotia begin to form, a process which is finished at about two weeks after sowing (Fig. 3 A, B).

***In vitro* growth.** Both isolates of the causal agent reached the edge of the agar plate within three days, the mean radial growth rate being 16.8 (SnO) and 16.4 mm/day (SnS), during days two and three. Under the same conditions, the isolate ScC (*S. cepivorum*) had an initial lag-phase of nearly one day and needed slightly more than four days to reach the edge of the plate, with a radial growth rate of 16.2 mm/day, during days three and four (Fig. 2). In another experiment, with ScA as representative of *S. cepivorum*, similar results were obtained (data not shown).

Fig. 1. A: symptoms of "freckles" on outer bulb scales of onion; B, D: details; C: cross section of sclerotium; E: symptoms on garlic bulb scales (experimental inoculation). Bar C: 200 µm; D: 1 mm.
Fig. 1. A: síntoma de "peca" en catáfilas externas de cebolla; B, D: detalle; C: corte de un esclerocio; E: síntoma en catáfilas externas de ajo (inoculación experimental). Barra C: 200 µm; D: 1 mm.

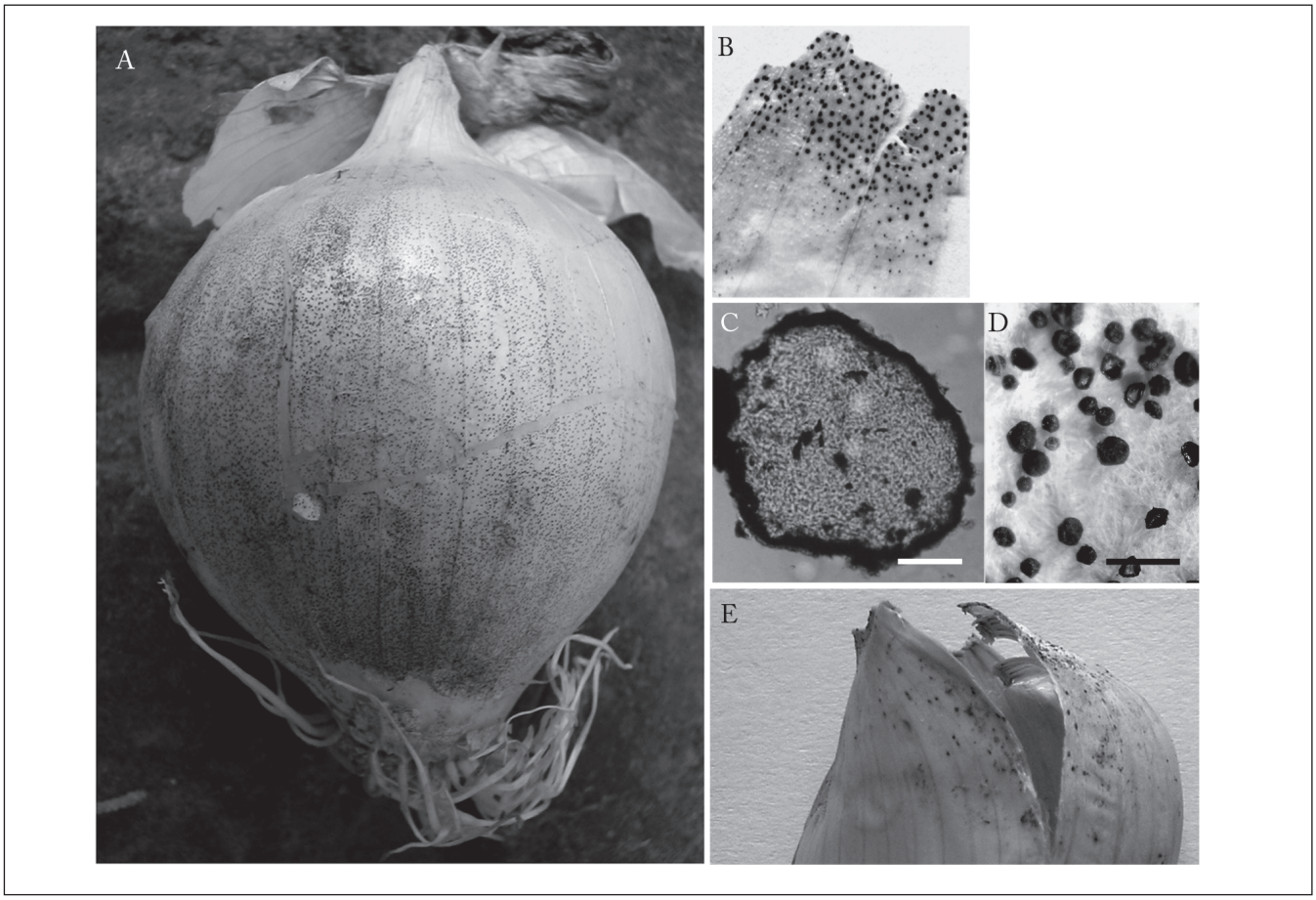
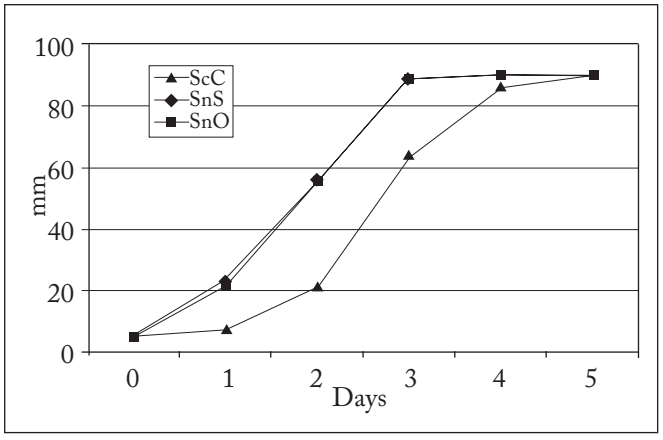
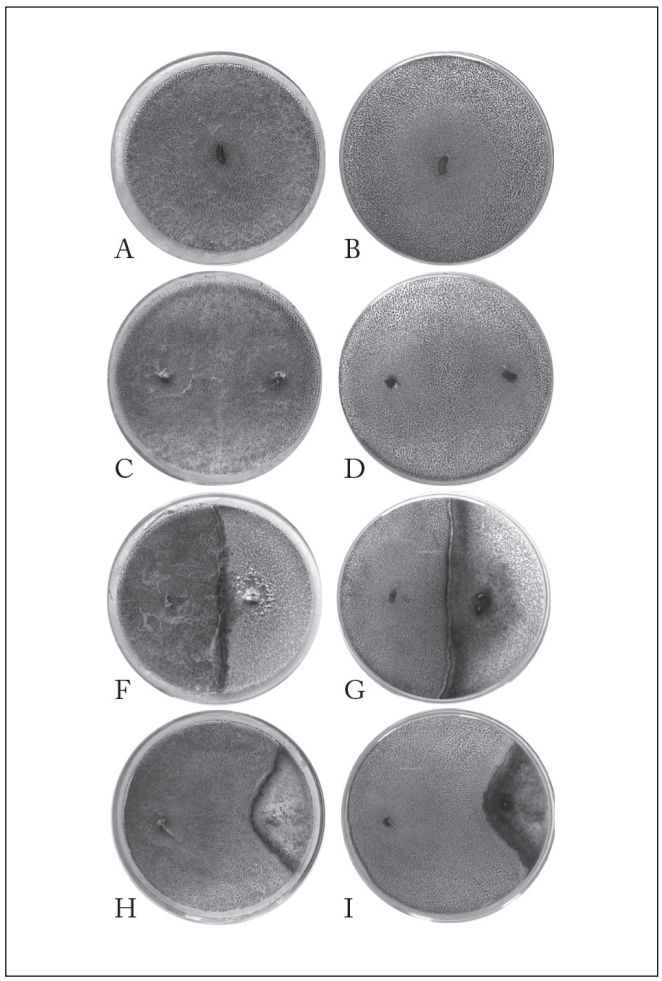


Fig. 2. Growth curves of colonies of isolates SnO and SnS (both *Sclerotium* sp.) and ScC (*Sclerotium cepivorum*) on PDA in 9 cm Petri dishes.
Fig. 2. Curvas de crecimiento de las colonias de los aislados SnO y SnS (*Sclerotium* sp.) y ScC (*Sclerotium cepivorum*) en APD en cajas de Petri (9 cm diámetro).



Mycelial compatibility testing. In many fungi, including *Sclerotium* spp., different mycelial compatibility groups (MCG) have been identified. The mycelia of isolates belonging to the same MCG merge forming one uniform colony when inoculated in the same dish, while mycelia of isolates of different MCGs either do not fuse and remain separated by a small white line or fuse and form a dark band of dead hyphae in the interaction zone (Earnshaw & Boland, 1997; Schafer & Kohn, 2006). As expected, in the self-self pairings (SnO/SnO; SnS/SnS) fusion of the mycelia (type A of Earnshaw & Boland, 1997) was observed, indicating their autocompatibility (Fig. 3 C, D). When the two isolates of the unknown fungus were paired (SnO/SnS) a light brown line indicating incompatibility formed in the interaction zone of the two mycelia [reaction type C of Earnshaw & Boland (1997)]. The pairings of the isolates of the unknown fungus with those of *S. cepivorum* (SnO/ScA; SnO/ScC; SnS/ScA; SnS/ScC) all yielded incompatible reactions with the formation of dark brown bands [type B of Earnshaw & Boland (1997)] where the colonies met (Fig. 3 E, H).

Fig. 3. Cultures of *Sclerotium* isolates on PDA in 9 cm Petri dishes; left: face view; right: bottom view. A, B: isolate SnS; C, D: self-self pairing SnSxSnS; E, F: self-nonself pairing SnSxScC; G, H: self-nonself pairing SnOxScA (for identification of isolates see text).
Fig. 3. Cultivos de aislados de *Sclerotium* en APD en cajas de Petri (9 cm); izq.: vista de arriba; der.: vista de abajo. A, B: aislado SnS; C, D: apareado homólogo SnSxSnS; E, F: apareado heterólogo SnSxScC; G, H: apareado heterólogo SnOxScA (identificación de los aislados ver en el texto).



Pathogenicity tests. *Experiment 1*: The inoculated onion bulbs developed the typical sclerotia on the dry outer scales, from their base up to the soil line, much the same as in naturally infected bulbs. Sclerotia were more numerous on the white cultivar than on the yellow one. A few sclerotia were also seen in the outer fleshy scales of the white cultivar. No differences were observed between the two fungal isolates studied or between the two temperature regimes. No signs or symptoms were seen on the control plants.

The inoculated plants were extracted carefully from the soil and after a few days of incubation in a humid chamber, some sclerotia developed externally on the roots without any disease symptoms. Such sclerotia were roundish, not flattened as those incrusted within the bulb scales. The original fungus could be reisolated from newly formed sclerotia.

Experiment 2: The inoculated onion seedlings developed some sclerotia on the roots and on the dry sheaths of the outer leaves. No signs or symptoms were seen on control plants. There were no significant differences between inoculated and control plants for survival (p=0.79), plant height (p=0.60) and root length (p=0.08), after two months.

Experiment 3: The inoculated garlic plants were carefully examined but only three of 12 plants inoculated with isolate SnO and two of 12 plants inoculated with SnS had developed sclerotia on the outer scales, indistinguishable from those formed in onion (Fig. 1 E). There were no other symptoms present. The isolate SnS was reisolated from the newly formed sclerotia. No sclerotia were found on the non-inoculated control plants.

DISCUSSION

Freckles is characterized by the formation of sclerotia in and on infected host organs. The shape and anatomical structure of sclerotia and the lack of sexual and mitosporic reproduction structures place the fungus in the form genus *Sclerotium*. The absence of clamp connections permits its provisional placement within the ascomyceteous fraction of this genus (Xu et al., 2010).

The *Sclerotium* sp. described here in some respects resembles *S. cepivorum*. Both fungi infect alliaceous hosts. Both produce sclerotia of similar size, shape and anatomy, *in vitro* and on the host. The hyphae are similar in diameter and branching pattern. However, there are also important differences. While *S. cepivorum* forms an abundant dense mycelium on the infected host tissue, the freckles agent does not; also on PDA, the mycelium of the former is more abundant than that of the latter. The two *S. cepivorum* isolates tested had an initial lag-phase when grown on PDA, while the two isolates of *Sclerotium* sp. had not. The latter fungus was shown to infect onion and garlic tissue and to produce sclerotia at temperatures between 25 and 30 °C; *S. cepivorum* is known to prefer lower temperatures and normally does not cause disease above 24 °C (Utkhede, 1982; Entwistle, 1990). Finally, *S. cepivorum* is an extremely aggressive pathogen able to cause rot in most of the host's organs and tissues, while *Sclerotium* sp. colonizes roots and outer bulb scales where only sclerotia appear as sign of infection and no other symptoms are seen.

Freckles is a new symptom on onion bulbs, only known on white-scaled cultivars. Although yellow-scaled onions and garlic could be infected experimentally, natural infections have not been noticed. The freckles agent is not able to kill onion plants, not even in the seedling stage, and does not produce rot or any other kind of tissue deterioration. The presence of small dots in the outer bulb scales might be considered, at

most, as a trivial cosmetic imperfection. Thus, we conclude that freckles is not a disease of any economic concern and *Sclerotium* sp. should not be considered a pathogen of onion. For the moment we are not able to define if the relationship of *Sclerotium* sp. to onion is parasitic, commensalistic or mutualistic. Whether or not this fungus is able to protect alliaceous crops against infection with *S. cepivorum* should be tested.

The fact that freckles is only known from the LRCV suggests that its causal agent might be a native element of the southern Pampas region. As the mycelial compatibility testing has shown that the two isolates of the new *Sclerotium* sp. belong to different MCGs it seems that there exists a certain degree of genetic variability in the field populations. Very recently, isolates of *Sclerotium* sp. obtained from garlic plants in Mendoza Province, Argentina, were shown to be non-pathogenic on garlic bulblets and onion seedlings (Arriagada & Valdez, 2011; Valdez & Arriagada, 2011; Valdez et al., 2011). It remains to be seen if those isolates are related to the freckles agent.

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