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## Research Papers

# Unraveling the infection process of garlic by *Fusarium proliferatum*, the causal agent of root rot

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**Summary.** Since the mid-2000s, and despite demanding production rules, *Fusarium proliferatum* (Matsushima) Nirenberg has been found on garlic heads during storage inducing root and bulbs rots. Brown spots on the surface of garlic cloves and water-soaking of heads were noted. Histological observations of the fungus during early stages of infection were made from clove to the cellular levels. *Fusarium proliferatum* germinates, colonizes roots and degrades the outer root and parenchyma cell layers 72 h post inoculation. Conidium germination and host colonization are facilitated by the emergence of garlic roots, creating cellular debris and natural wounds. Hyphae of the pathogen did not penetrate healthy host cells and appeared to degrade them before penetration. These results provide understanding of when and how quickly *F. proliferatum* penetrates garlic bulbs. This is a primary step towards elucidating the life cycle of this pathogen during the garlic drying process, and development of an efficient and sustainable bulb rot management strategy.

**Keywords.** Histology, electron microscopy, garlic rot, host-parasite interaction.

## INTRODUCTION

From Hippocratic medicine (Totelin 2015) to modern and sophisticated molecular cuisine (This 2006), garlic has been used throughout history and has a special place in human civilization. World garlic production reached a peak of more than 26 million tons produced in 2016, rising from 11 million in 2000 (FAOSTAT, 2018). Global average price is \$US 2.35 kg<sup>-1</sup> and continues to increase (Tridge, 2019). China produces and exports the most of the world's garlic (80% of world production and 25 millions of tons per year).

France ranks 6th among European producers of garlic, with 21,000 tons produced each year in two main areas of cultivation: the South-East and the South-West of the country. Quality is an important objective for French producers in that French garlic benefits from high quality standards and certification, which ensure control of production such as the restricted geographic areas of production (one AOP – Appellation d'Origine Protégée – and four IGP – Indication Géographique Protégée – labels) (INAO, 2019). For the seed

certification, garlic production in each field must be separated by 5 years, in order to reduce propagation of the nematode *Ditylenchus dipsaci* (Robert and Matthews 1995) and white rot caused by *Sclerotium cepivorum* (Basallote-Ureba and Melero-Vara 1993). All certified varieties are free from *Onion yellow dwarf virus* (OYDV) and *Leek yellow stripe virus* (LYSV), and have been obtained by meristem culture. In the field, all plants that show differences compared to the source material are destroyed. During all production steps before and after storage, damaged garlic heads are destroyed. Despite these control and certification standards for seed and plants, *Fusarium* rot of garlic emerged in France in approx. 2006 (Ricard 2017). During storage, browning of cloves commences from the basal plates and extends to the tops of the heads. Tissues become soft and water-soaked before complete rotting. Losses are variable: less than 1% in 2006 year, increasing to 25% on average in 2015. Some plots have been more affected than the others (from 1 to 80%), and high losses drastically reduce the volumes sold and therefore the income for producers. In extreme cases, farmers have ceased producing several varieties that were highly susceptible to root rot.

Eight *Fusarium* species have been found on symptomatic garlic, with the majority being *F. proliferatum* followed by *F. oxysporum* and then *F. solani* (Koleva 2004; Stankovic *et al.*, 2007; Ochoa-Fuentes *et al.*, 2012; Moharam *et al.*, 2013; Delgado-Ortiz *et al.*, 2016; Ignjatov *et al.*, 2017). The disease caused by *F. proliferatum* on garlic is common wherever the crop is grown, and was first reported in 2002 in Germany (Seefeldt *et al.*, 2002) and in 2003 in North America (Dugan *et al.*, 2003). The number of countries affected by *F. proliferatum* on garlic has increased to include Serbia in 2007 (Stankovic *et al.*, 2007), India in 2012 (Sankar and Babu 2012) and Egypt in 2013 (Moharam *et al.*, 2013). In France, the pathogen was recently identified as *F. proliferatum* (Matsushima) Nirenberg in 2018 (Leyronas *et al.*, 2018). *Fusarium proliferatum* is within the *F. fujikuroi* species complex native to Asia (O'Donnell *et al.*, 2013), and is responsible for bulb, root or fruit diseases on many crop plants, including onion (Toit *et al.*, 2003), soybean (Diaz Arias *et al.*, 2011), chive (Yamazaki *et al.*, 2013), lily (Lebiush-Mordechai *et al.*, 2014), welsh onion (Alberti *et al.*, 2017), peach (Xie *et al.*, 2018) and strawberry (Borrero *et al.*, 2019).

There is no officially accepted prophylactic or chemical control method for *Fusarium* spp. on garlic in France. One of the hurdles to developing control methods is the lack of knowledge of the pathogen infection processes into garlic bulbs. Mycelium starts to develop around the basal plates of bulbs where the roots emerge

(Stankovic *et al.*, 2007; Tonti *et al.*, 2012). Fungal growth also occurs on the bulb apices, where the skin cracks as leaves emerge. Then tissues become brown, and wilt from the bottom to the top at a regular rate. Wilting also starts from wounds on the bulb surfaces. Microscope observations have been made of infection of sorghum plants, showing that hyphae of *F. proliferatum* quickly penetrate (about 2 weeks after sowing) the endodermal and xylem parenchyma layers of roots, colonize complete root cortices (Ndambi *et al.*, 2012).

The objectives of the present study were to investigate the infection processes of garlic by *F. proliferatum*, and in particular to determine when and where the pathogen conidia enter host tissues. Observations were focused on the basal parts of the garlic heads, including the roots and tissues around the heads and the basal plates. Using light and electron microscopy, interactions were observed at different scales, from overall aspects to histological levels.

## MATERIALS AND METHODS

### *Preparation of biological material*

#### Biological material

A *F. proliferatum* strain (FA3-E01) isolated from pink garlic cloves cultivated in France during the summer 2017 was used in this study. This strain was previously purified and added to a laboratory fungal collection, and this strain has been shown to be aggressive on garlic bulbs (Leyronas *et al.*, 2018).

All garlic cloves used in this study were from a single lot of pink garlic from the south of France harvested in late June 2018. The cloves were all asymptomatic at the time of inoculation.

#### Preparation of conidium suspensions

Inoculum was produced on potato dextrose agar medium (Difco Laboratories) at 21°C under cool white light (12 h photoperiod, 23.8  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ). Mycelium plugs were taken from 1-week old cultures and were added on 250 mL capacity Erlenmeyer flasks containing 150 mL of potato dextrose broth (Difco Laboratories, Detroit). The flasks were placed on a rotary shaker at 100 rpm at 21°C under cool white light (12 h photoperiod, 23.8  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ). After 7 d, the medium was filtered through etamine filters (25–35  $\mu\text{m}$  pores) to remove mycelium fragments and retain microconidia. The concentration was evaluated with a haemocytometer, and

adjusted to  $1.0 \times 10^6$  conidia  $\text{mL}^{-1}$  using sterile deionized water. This concentration was known to recreate symptoms on cloves.

#### Inoculation of garlic cloves

Healthy peeled cloves of pink garlic ( $n = 6$ ) were surface-disinfected with 1% NaOCl for 1 min and rinsed in 3 successive baths of sterile water. Disinfected cloves were soaked in 200 mL of conidial suspension (or sterile water for negative control) inside a beaker placed on a rotary shaker at 100 rpm for 24 h at 21°C. The cloves were then placed inside a sterile plastic box at 23°C with saturated humidity and constant obscurity. Samples were collected after 6, 12, 24, 48 and 72 h for microscopic analyses.

#### Observations of samples at different scales

##### Sample preparation for light microscopy

All samples were first observed with an illuminated binocular magnifier. General structures of cloves, basal plates and tissues were described.

Control (uninoculated) and inoculated garlic cloves were longitudinally sliced and fixed in FAA (1/1/8, V/V/V, 37% formalin/glacial acetic acid/90% ethanol). To promote penetration of the fixative products, samples were subjected to vacuum for 20 min. After 48 h of fixation, the specimens were rinsed in distilled water and stored in 70% ethanol at 4°C until required. They were then dissected to collect 5 x 5 mm basal fragments that were processed for cytohistology. Briefly, the samples were dehydrated in a graded ethanol series (80-100%) and infiltrated in methacrylate resin (Kit Technovit 7100, Heraeus-Kulzer GmbH), according to the manufacturer instructions). Sections (3  $\mu\text{m}$  thick) were serially cut using a retraction microtome (Supercut 2065, Reichert-Young), and collected on microscope slides. For routine observations, sections were stained by toluidine blue, a metachromatic dye (Clark, 1981). For each treatment, sections were stained to visualize major cell components using periodic acid-Schiff's reagent (PAS) for polysaccharides (starch and cellulose) (pink) and naphthol blue-black (NBB) for protein (blue) (El Maâtaoui and Pichot 1999). All microscope analyses were performed using a Leica DMR photomicroscope equipped for bright field, dark field, phase contrast and UV illuminations. Images were captured using a Leica DFC 300 FX digital camera and processed using Leica LAS software. For each treatment, approx. 60 serial sections from

five different garlic cloves were analyzed, and representative images were selected to illustrate the major histological traits. Attention was paid to initiation of infection, mycelium progression in clove tissues, and cyto-pathological effects.

##### Sample preparation for scanning electron microscopy

Garlic tissue samples were fixed in 2.5% (v/w) glutaraldehyde for 1 h and then washed three times with 0.2M sodium cacodylate buffer (pH 7.2). The samples were then dehydrated with a series of ethanol baths of increasing concentration (30% to 100%). The ethanol was replaced by HMDS until it completely invaded the samples and evaporated. After drying, the samples were mounted on aluminium stubs, sputter-coated with a layer of gold, and then observed with an FEI/Philips XL30 scanning electron microscope at 10 kV.

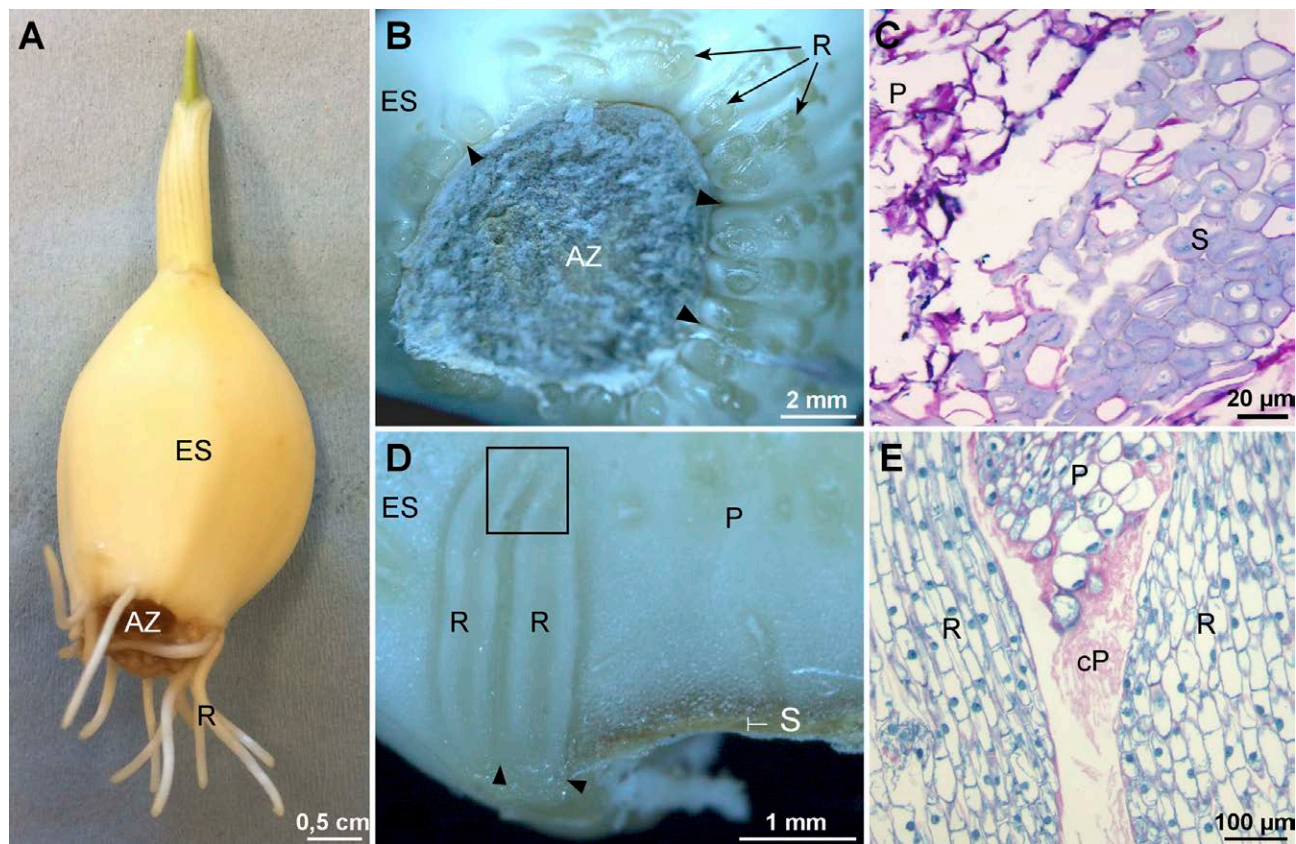
## RESULTS

#### Structure of healthy garlic tissues

Each peeled garlic clove usually had bulbil-like morphostructure, with a basal plate supporting a fleshy external leaf or scale (Figure 1A) attached to a short stem (not shown) that bore numerous root primordia (Figure 1B). After clove germination, root emergence took place between the external scale and the abscission zone of the basal plate, thus enlarging the space at the junction between the external scale and the abscission zone. Microscope analyses of sections from the abscission zone showed that this area was composed of thick-walled, dead cells (Figure 1C). Condensation and retraction of cytoplasm and degradation of chromatin inside nuclei indicated that the cells were dying. Inside cloves, roots embedded in leaf parenchyma (Figure 1D) created each an aperture and many dead collapsed cells while emerging (Figure 1E).

#### Progression of *Fusarium proliferatum* in cloves

After 72 hpi, hyphae adhered and colonized the junctions between roots and parenchyma of external scale edges (Figure 2A). Inoculated conidia germinated on the surface of epidermis cells (Figure 2B). Mycelium then invaded the entire area composed of the parenchyma of the external scale and suberized tissue (Figure 2C). Abscission zones (AZ) were covered with white mycelium (not shown). Microscope analyses of these areas



**Figure 1.** Morphology and structure of healthy garlic cloves. A, Peeled clove after 3 d in sterile distilled water. B, Abscission zone. C, Section of the abscission zone. D, Longitudinal section of the basal part of an ungerminated clove. E, Section from the area indicated by a square in D. ES: external scale, AZ: abscission zone, R: roots, P: parenchyma, S: thick-walled cells, cP: collapsed parenchymatous cells. Black arrowheads indicate junctions between roots and between AZ boundaries and root primordia. A, B and D, Stereomicrographs. C and E, Light micrographs of PAS/NBB stained samples.

showed dense mycelium growth in the cellular debris close to suberized tissue (Figure 2D). On each clove, a coat of white hyphae appeared all around the roots from the base to the top and at the edge of the external scale (Figure 2E). Specific infection structures were not observed. *Fusarium proliferatum* proliferated as mycelium in a saprophyte-like manner.

Browning of cloves progressed from the base to the top at 72 hpi. The boundary between healthy and diseased tissues was easily discernible, and mycelium entirely covered roots (Figure 3A). At 48 hpi, germinated conidia and mycelium were observed against de-structured suberised regions (Fig. 3B). The pathogen was also observed to be growing inside collapsed dead cells from the external clove scales resulting from the root emergence (Figure 3C). At 72 hpi and beneath the mycelium coat, root tissues were degraded and hyphae penetrated only inside the first layers of dead cells (Figure 3D).

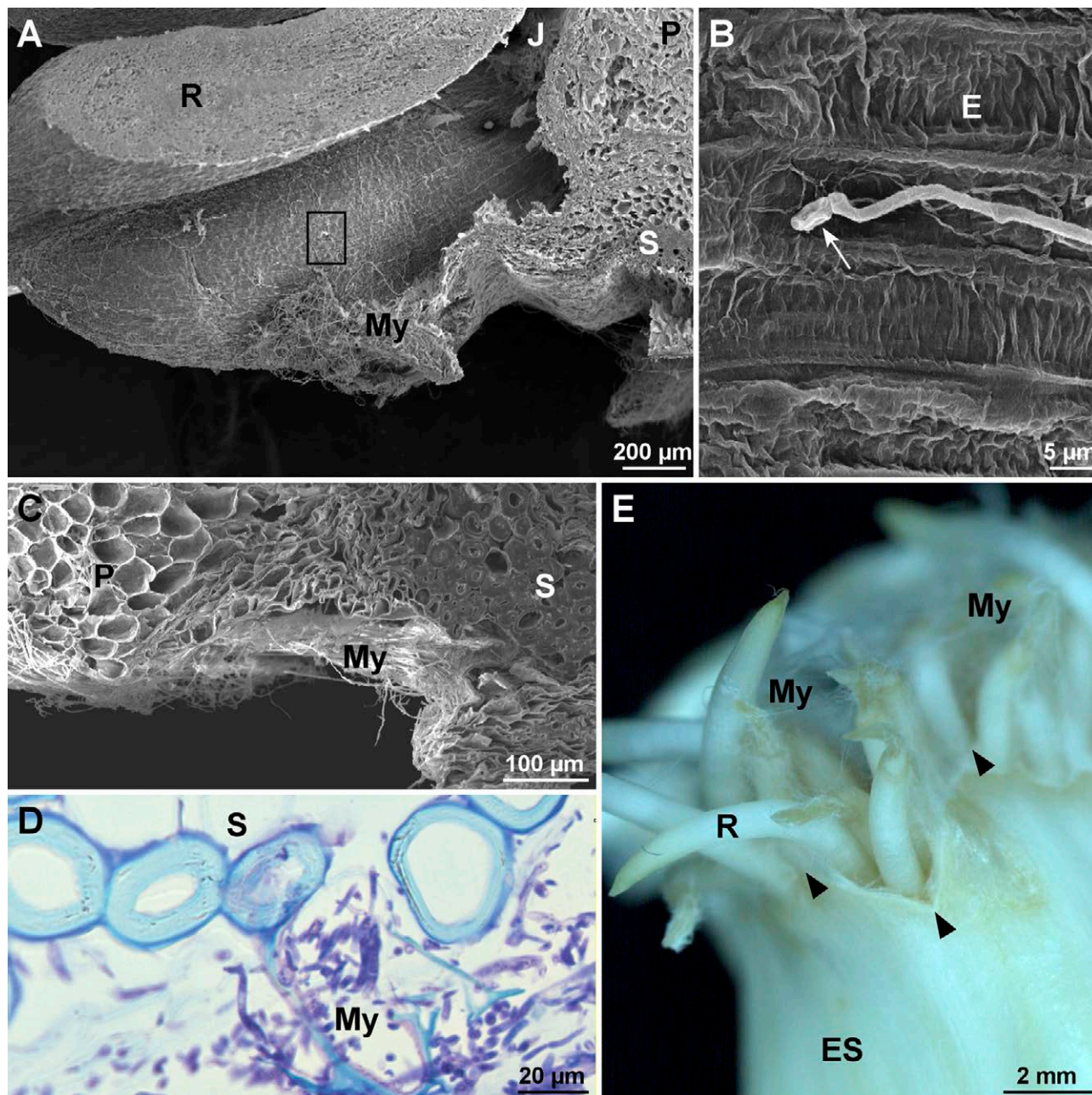
#### De-structuring of clove cell layers

In tissues infected by *F. proliferatum*, the cells died and shrank particularly within the parenchyma of external scales and in the superficial layers of the roots (Figure 4A and B). Three levels of degraded tissue could be discerned; entirely degraded tissue, collapsed cells filled with secondary metabolites (deep blue) and dead matrices of cells filled with fungal mycelium (III), a border between dead and healthy cells (II) and healthy parenchyma cells (I) (Figure 4B). The dead cells accumulated proteins, as indicated by the azure colour of their contents.

#### DISCUSSION

This study has clarified the pathway by which *F. proliferatum* enters garlic cloves. *Fusarium* species (e.g. *F. oxysporum*, *F. solani* and *F. verticillioides*) are well

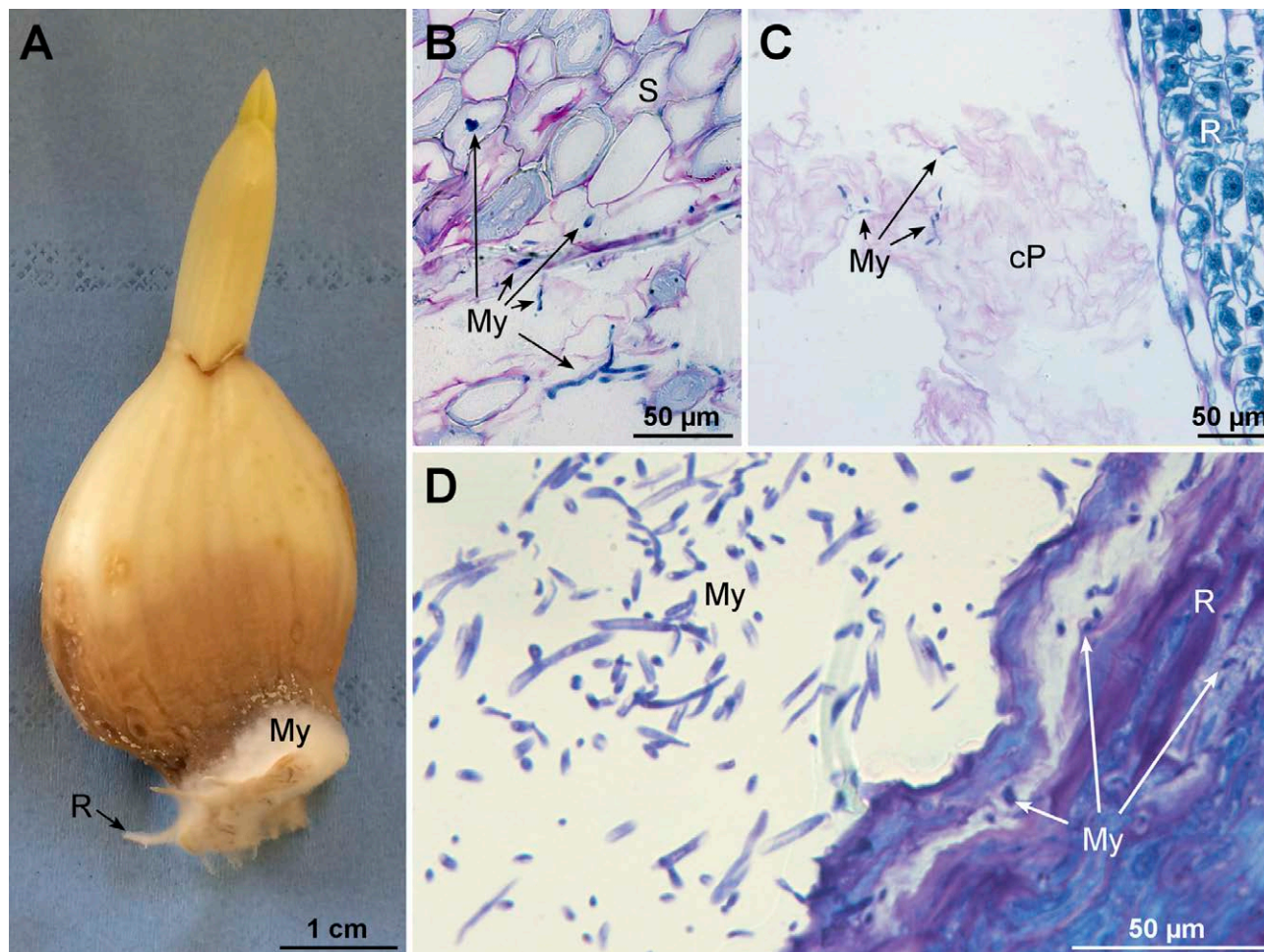




**Figure 2.** Mycelium development of *Fusarium proliferatum*. A, SEM micrograph of a germinating garlic root. B, High magnification of the area indicated by the square in A. C, Section of the basal part of an infected clove. D, Detail of suberized tissue. E, Basal part of a clove 72 h post inoculation. R: roots, J: junction, P: parenchyma, My: mycelium, E: epidermis, S: thick-walled cells, ES: external scale. White arrow in B indicates a germinated conidium. Black arrowheads in E indicate junctions between roots and between the abscission zone boundary and root primordia. A and B, Scanning electron micrographs. D, Light micrograph after TB staining. E, Stereomicrograph.

known for their capacity to infect vascular and root tissues causing wilt on plants (Agrios, 2005). In the present study, rapid degradation of garlic root cells was observed after inoculation with *F. proliferatum*, but the pathogen did not invade host conductive tissues. *Fusarium pro-*

*liferatum* progressed from the points of infection in all directions. Proliferation of the fungus in garlic tissue was reduced in the presence of thick-walled cells, and was directed toward the parenchyma. Although this pathogen possesses the enzymes required to degrade



**Figure 3.** Morphology and cytohistological aspects of *Fusarium proliferatum* infected garlic cloves 48–72 h post inoculation. A, Image of an infected clove. B and C, Micrographs illustrating the progression of clove infection 48 h post inoculation. D, Detail of the decayed root tissue. R: roots, My: mycelium, S: thick-walled cells, cP: collapsed parenchyma cells. A, Stereomicrograph. B and C, Light micrographs after PAS/NBB staining. D, Light micrograph with TB staining.

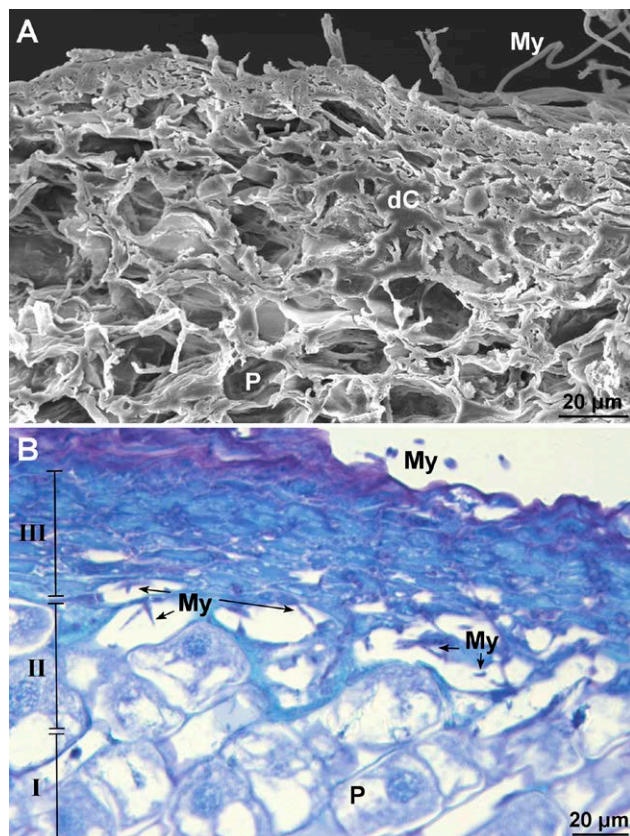
suberized cells, such as laccases (Regalado *et al.*, 1999; Hernández Fernaud *et al.*, 2006), chitinases, glucosidases and galactosidases (Keshri and Magan 2000), *F. proliferatum* probably takes the path of least host resistance.

*Fusarium proliferatum* rapidly infected garlic root tissues before infecting clove tissues. Germination of roots may have triple positive indirect effects on the development of the root rot. First, while emerging, roots degrade parenchyma cells and release cellular debris that may be utilized for growth of the fungus. Second, the physical barrier of the host epidermis breaks and allows penetration of the pathogen into host tissues. In some preliminary experiments, we have observed that *F. proliferatum* was unable to enter through intact garlic epidermis surfaces. Third, the presence of host and non-host roots in the soil are known to induce germination

of fungal conidia and attract hyphae of *F. oxysporum* (Nelson 1981). Further studies could assess if this is also the case for *F. proliferatum* and garlic roots.

In the present study, *F. proliferatum* was observed to colonize decayed host tissues, but not to penetrate healthy cells. Tissues destroyed beyond the margins where fungal growth occurred were seen, indicating that the fungus deployed strategies, such as enzymes and toxin production, to destroy host tissues prior to colonization. The limits between dead and living cells were clearly visible. This could indicate activity of the many organosulfur compounds produced and stored inside garlic cells. These molecules are responsible for the characteristic fragrance of garlic. These compounds, such as alliin, have been widely studied as potential biocontrol agents against fungi, bacteria and





**Figure 4.** Histopathological effects of *Fusarium proliferatum* on infected clove tissues. A and B, Detail of the first root cells layers 72 h post inoculation. My: mycelium, dC: dead cells, P: parenchyma. A, Scanning electron micrograph. B, Light micrograph after TB staining.

other pests (Curtis *et al.*, 2004). Allicin is synthesized from a precursor molecule (alliin) through the action of alliinase activated when cells are damaged. The fact that *F. proliferatum* is able to grow, colonize and develop on a matrix containing organosulfur compounds was interesting. Previous studies have found that garlic extracts inhibit *F. proliferatum* on culture media at pH 3 to 7 (Chen *et al.*, 2018). One hypothesis to explain pathogenicity is the production of desulfurization enzymes (sulfatases) by some *F. proliferatum* strains (Shvetsova *et al.*, 2015). This overcoming of host resistance could also be linked to glutathione metabolism (Leontiev *et al.*, 2018). Another example of fungi being able to grow in the presence of organosulfur is *Coriolus versicolor* known to degrade this type of compound in wood (Linder 2018). One hypothesis about the emergence of root rot of garlic is that some pathogen strains may have acquired resistance to garlic compounds. In further studies, we will assess the impacts of garlic

extracts on *in vitro* development of several *F. proliferatum* strains, and evaluate the ability of *F. proliferatum* strains, collected from other crops, to develop disease symptoms on garlic.

Like most *Fusarium* spp., *F. proliferatum* has a soil-borne phase. The present study showed that this fungus can penetrate garlic cloves at the outline/contour of their basal plates when the roots emerge. This indicates that *F. proliferatum* remains dormant in the soil, is attracted to germinating garlic cloves (at early stages of their development), and then penetrates through wounds without generating visible symptoms. Airborne phases cannot be excluded. *Fusarium proliferatum* is a prolifically sporulating species, and in Spain, conidia collected in rainwater were shown to be aggressive on garlic (Gil-Serna *et al.*, 2016). In the field, sprouting garlic leaves take funnel shapes, and rainwater is directly led to germinated cloves where the epidermis is weakened. In further experiments, it could be interesting to test independent inoculation of shoots and of the basal plates could be investigated. Knowledge of the origin of inoculum that induces rot on garlic is crucial for development of efficient, sustainable and environmentally-friendly root rot management strategies.

Through of French and European actions, French garlic producers are encouraged to reduce their use of phytosanitary products (European Directive 2009/128/EC). There is a need for efficient alternative disease control methods, among which are aimed at management of garlic root rot. Soil solarization could be explored to control the soil-borne population of *Fusarium* spp. In Japan, this method was efficient for control *Fusarium* wilt of strawberry caused by *F. oxysporum*, in fields and greenhouses (Koike and Gordon 2015). Results could be linked to heat alone or to selection of antagonistic soil microorganisms. In another research, solarization with *Medicago sativa* amendment was shown to be an efficient, non-chemical, method for control of *Fusarium* wilt of cucumber caused by *F. oxysporum* (Yao *et al.*, 2016). Although soil solarization has proved to be efficient against species other than *F. proliferatum*, it would be worth testing this method for garlic crops. The use of biocontrol agents such as bacteria applied on garlic cloves may also be promising. Four strains of *B. subtilis* have been shown to reduce severity of disease caused by *F. proliferatum* strains on garlic (Bjelic *et al.*, 2018).

Biocontrol and solarization need to be applied at the right time, in optimum conditions, to have appropriate activity during or before crop planting. To this end, some of our current studies are focusing on the impacts of abiotic factors on *F. proliferatum* mycelium growth and sporulation.



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