Quantitative and Microscopic Assessment of Compatible and Incompatible Interactions between Chickpea Cultivars and *Fusarium oxysporum* f. sp. *ciceris* Races

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Abstract

**Background:** Fusarium wilt caused by *Fusarium oxysporum* f. sp. *ciceris*, a main threat to global chickpea production, is managed mainly by resistant cultivars whose efficiency is curtailed by *Fusarium oxysporum* f. sp. *ciceris* races.

**Methodology:** We characterized compatible and incompatible interactions by assessing the spatial-temporal pattern of infection and colonization of chickpea cvs. P-2245, JG-62 and WR-315 by *Fusarium oxysporum* f. sp. *ciceris* races 0 and 5 labeled with ZsGreen fluorescent protein using confocal laser scanning microscopy.

**Findings:** The two races colonized the host root surface in both interactions with preferential colonization of the root apex and subapical root zone. In compatible interactions, the pathogen grew intercellularly in the root cortex, reached the xylem, and progressed upwards in the stem xylem, being the rate and intensity of stem colonization directly related with the degree of compatibility among *Fusarium oxysporum* f. sp. *ciceris* races and chickpea cultivars. In incompatible interactions, race 0 invaded and colonized ‘JG-62’ xylem vessels of root and stem but in ‘WR-315’, it remained in the intercellular spaces of the root cortex failing to reach the xylem, whereas race 5 progressed up to the hypocotyl. However, all incompatible interactions were asymptomatic.

**Conclusions:** The differential patterns of colonization of chickpea cultivars by *Fusarium oxysporum* f. sp. *ciceris* races may be related to the operation of multiple resistance mechanisms.

Introduction

Chickpea (*Cicer arietinum* L.) is a major source of human food and animal feed, and one of the world’s most important pulse crops after dry beans (*Phaseolus vulgaris* L.) and dry peas (*Pisum sativum* L.) [1]. Chickpeas are grown throughout tropical, subtropical and temperate regions of the world [1]. Fusarium wilt, caused by the soilborne fungus *Fusarium oxysporum* Schlechtend.: Fr. f. sp. *ciceris* (Padwick) Matuo & K. Sato, is one of the most important diseases limiting chickpea production worldwide. Annual yield losses from this disease have been estimated to range from 10 to 15%. However, locally Fusarium wilt epidemics can cause 100% loss under disease-favorable conditions [2,3,4].

Fusarium wilt in chickpea is best managed using resistant cultivars [5]. However, the effectiveness of this management strategy can be curtailed by the high pathogenic variability in *F. oxysporum* f. sp. *ciceris* populations [6]. Two pathotypes (yellowing and wilting) and eight pathogenic races (races 0, 1A, 1B/C, 2, 3, 4, 5, and 6) have been described to date [7,8,9]. The yellowing pathotype induces progressive foliar yellowing with vascular discoloration and late plant death, while the wilting pathotype causes fast and severe chlorosis, flaccidity, vascular discoloration, and early plant death. For both syndromes, the subterranean tissues of affected plants show no external symptoms.

Pathogenic races of *F. oxysporum* f. sp. *ciceris* differ in geographic distribution. Races 0, 1B/C, 5, and 6 are found in the Mediterranean Basin and California (USA) [3,6,9,10]. Race 1A has been reported in the Indian subcontinent [2], California, and the Mediterranean Basin [6,9,10], whereas races 2 and 3 have been reported in Ethiopia, India, and Turkey [2,11,12] and race 4 has only been reported in Ethiopia and India [2,12]. Races 0 and 1B/C induce the yellowing syndrome, whereas races 1A through 6 cause the wilting syndrome [6,9]. Knowledge of the geographical distribution of *F. oxysporum* f. sp. *ciceris*-races is critical to disease management, because individual races vary in their interaction
with differential chickpea lines as well as in the amount of inoculum needed to induce a given amount of disease in susceptible chickpeas [13,14].

Our understanding of the cellular basis of host-pathogen interactions underling vascular wilt diseases has been much improved due to the use of confocal laser scanning microscopy (CLSM) and fluorescent protein-tagged pathogen isolates. This approach has allowed 'in situ' time-lapse imaging of plant-pathogen interactions, thus avoiding concerns associated with fixing plant tissues for light and electron microscopy observations [15,16]. Thus, use of CLSM has provided better understanding of the infection process of F. oxysporum in multiple hosts: i) the ability of F. oxysporum f. sp. ciceris race 4 to directly penetrate epidermal cells of banana roots, and the role of the epidermal cells of root caps and elongation zone as potential sites of ingress [17]; ii) the root meristem of emerging lateral roots and primary root tips of Arabidopsis thaliana are infection courts for a wilting F. oxysporum isolate, regardless the level of resistance of the host ecotypes [18]; iii) the pattern of Medicago trunculata colonization by F. oxysporum f. sp. medicaginis is similar between susceptible and tolerant lines [19]; iv) differential virulence of F. oxysporum f. sp. phaseoli isolates on bean is directly correlated with the speed of xylem vessels colonization [20]; vi) resistant and susceptible melon lines are differentially colonized by F. oxysporum f. sp. melonis race 1.2 [21]; or vii) the infection of tomato plants by F. oxysporum f. sp. lycopersici is initiated by development of a hyphal network on the upper part of taproot, followed by the growth of hyphae towards the elongation zone, lateral roots and root apices [22].

Pathogenic races of F. oxysporum f. sp. ciceris differ significantly in the amount of inoculum needed to induce a given amount of disease in susceptible chickpeas [13,14]. For example, the inoculum density of the least virulent race 0 needs to be ~100 times greater than that of the highly virulent race 5 in order to cause the same amount of disease [13,14]. Characterization of the underlying cause of differential virulence by different races will help understand how different chickpea genotypes interact with pathogenic races of F. oxysporum f. sp. ciceris. However, a few studies that have characterized the Fusarium wilt/chickpea pathosystem focused mainly on compatible interactions [23,24,25]. Therefore, additional studies are needed for better understanding the mechanisms underlying incompatible interactions.

In this present research, we hypothesized that the nature of F. oxysporum f. sp. ciceris races influences the processes of infection and colonization of resistant chickpeas by the pathogen, and addressed this hypothesis by determining the preferential infection court and quantitatively assessing the spatial-temporal patterns of colonization in both compatible and incompatible interactions with host cultivars differing in the level of resistance to F. oxysporum f. sp. ciceris races. To support this objective, isolates of two races (0 and 5) were first transformed with a ZsGreen fluorescent protein [18]. Then, one transformant of each race was selected based on growth characteristics, pathogenicity, and virulence phenotypes in comparison with the corresponding wild parent isolates. Finally, three cultivars that differentially respond to these races were inoculated to describe and quantify the pattern and dynamics of colonization.

Materials and Methods

Growth, Storage, and Genetic Transformation of F. oxysporum f. sp. ciceris Isolates

Two F. oxysporum f. sp. ciceris isolates (Foc-7802, race 0 and yellowing pathotype and Foc-W6-1, race 5 and wilting pathotype) were transformed with a construct encoding the ZsGreen fluorescent protein using the procedures described by Khang et al. [26]. Coding ZsGreen protein fragment was cloned in Xhol-HindIII sites of pBHt2 [27] to produce the binary vector SK2241. Agrobacterium tumefaciens strain EHA105 [28] was used to transform the isolates. Six and 11 transformants were obtained from Foc-7802 and Foc-W6-1, respectively. All transformants were grown on Fusarium minimal medium [29] amended with hygromycin B (50 μg ml⁻¹). To check for the successful expression of ZsGreen, single-spored cultures of transformants were examined with a confocal laser microscope (Nikon TE. 2000-S, Melville, NY, USA). Four transformants of each race were then chosen for subsequent phenotypic characterization.

The wild-type strains and their transformants were deposited in the culture collection of the Department of Crop Protection, Institute for Sustainable Agriculture (IAS-CSIC), Córdoba, Spain.

All cultures were single-spored before long-term storage in sterile soil tubes at 4°C and in 35% sterile glycerol in water at ~80°C. Active cultures were started by placing small aliquots of soil cultures onto a plate containing fresh potato dextrose agar (PDA; Difco Laboratories, Detroit, MI) and incubating for 4 days at 25°C and a 12-h photoperiod of a mixture of fluorescent white and near-UV light at 36 μE m⁻² s⁻¹.

Phenotypic Characterization of Transformants

Colonies morphology, mycelial growth rate, pathogenicity, and race phenotype of the selected transformants were compared with those of the corresponding wild-type strains. To compare for colony morphology and mycelial growth rate, a PDA plug of actively growing culture was placed onto PDA plates and incubated at 25±1°C and a 12-h photoperiod (fluorescent white and near-UV light at 36 μE m⁻² s⁻¹). Three replicates (plates) for each of the transformants and wild-type strains were arranged in a completely randomized design. The radial mycelial growth (RMG) was determined daily by measuring the length of four radii. The radial growth rate (RGR) was calculated by the slope of the linear regression of the mean colony radius over time. Mycelial pigmentation, the shape of colony margin, and any feature that looked different from wild-type strains were recorded.

Pathogenicity and race phenotypes of the transformants were assessed by inoculating chickpea cultivars that differ in their reaction to races 0 and 5, including cvs. P-2245 (susceptible to both races), PV-1 (susceptible to race 0 and resistant to race 5), JG-62 (resistant to race 0 and susceptible to race 5), and WR-315 (resistant to both races). Inocula were prepared in Erdemeyer flasks containing 400 g of a cornmeal-sand mixture (CMS) infested by the fungus, and mixed with a sterile potting mix as described before [14]. Plants were grown and infected at 10° cu g soil mix⁻¹ in 15-cm diameter clay pots (four plants per pot) in a walk-in growth chamber adjusted to 25±2°C and a 14-h photoperiod (fluorescent white light at 360 μE m⁻² s⁻¹) for 42 days. There were four replicated pots per isolate arranged in a completely randomized design. Incidence (I, 0 to 1 scale), and severity of foliar symptoms [S, rated on a 0 to 4 scale according to the percentage of affected foliage: 0 = 0%, 1 = 1–33%, 2 = 34–66%, 3 = 67–100%, 4 = dead plant] were assessed on individual plants at 2- to 3-day intervals. Disease intensity index (DII) was obtained using the following: DII = (I × S)/4 [14]. Disease progress curves were obtained from the accumulated DII over time from the date of inoculation.

Cytological Characterization of Compatible and Incompatible Interactions via Confocal Laser Microscopy

Two ZsGreen-tagged transformants of races 0 and 5, named as F11 and F93, respectively, were used to infect chickpea cvs. P-2245, JG-62, and WR-315, which differ in disease reaction to
Incompatible Interactions via Confocal Laser Microscopy

Cytological Characterization of Compatible and Incompatible Interactions via Confocal Laser Microscopy

No substantial differences were observed during the initial stages of infection of the tap root and root hairs by races 0 and 5 (Fig. 1). However, the spatial-temporal dynamics of subsequent infection processes of chickpea plants varied considerably among the *F. oxysporum* f. sp. *ciceris* race/chickpea cultivar combinations studied here (Figs. 1, 2, 3, 4, Table 2).

For race 0, the earliest infection event consisted of the development of a dense hyphal network without pattern on the surface of the tap root, including the root cap, apical meristem and subapical zones, which took place during the first 4 days after inoculation (dai) (Figs. 1a,b, 2a–c, Table 2). The fungus also grew on the surface of lateral roots in a manner similar to that observed on the taproot with the apical zone being heavily colonized by hyphae that grew along anticlinal wall junctions of epidermal cells (Fig. 1c–e). Following the surface growth, the hyphae directly penetrated epidermal cells without forming specialized penetration structures (Figs. 1f, 2d–f). By 6 dai, the race 0 transformant was found in the intercellular spaces of the outermost cell layers of the root cortex of cvs. P-2245 (compatible interaction) and JG-62 (incompatible interaction) (Figs. 2g–h, 3a,b), but it remained restricted within the root epidermis of cv. WR-315 (incompatible interaction) (Figs. 3c–d).
### Table 1. Characterization of two isolates of *Fusarium oxysporum* f. sp. *ciceris* (races 0 and 5) transformed with the ZsGreen fluorescent protein.

| Isolatesa | Mycelial growthb | Elements of the disease progress curvec |
|-----------|------------------|----------------------------------------|
|           | RMGfinal (mm)    | RGR (mm/h) | P-2245  | JG-62  | PV-1  | WR-315 | P-2245  | JG-62  | PV-1  | WR-315 |
| F. oxysporum f. sp. ciceris race 0 | | | | | | | | | | |
| F3       | 36.3             | 0.18       | 1       | 0.00  | 0.00  | 0.76   | 0.00  | 0.81  | 0.00  | 16.37* – | 16.50* – |
| F8       | 36.3             | 0.18       | 1       | 0.00  | 0.00  | 0.75   | 0.00  | 0.84  | 0.00  | 16.87* – | 16.50* – |
| F11      | 35.8             | 0.18       | 1       | 0.00  | 0.00  | 0.71   | 0.00  | 0.81  | 0.00  | 20.12 – | 18.87 – |
| F12      | 35.9             | 0.18       | 1       | 0.00  | 0.00  | 0.73   | 0.00  | 0.83  | 0.00  | 19.40 – | 17.43* – |
| Foc-7802(wt) | 36.2           | 0.18       | 1       | 0.00  | 0.00  | 0.71   | 0.00  | 0.84  | 0.00  | 20.00 – | 20.30 – |
| F. oxysporum f. sp. ciceris race 5 | | | | | | | | | | |
| F73      | 29.3*            | 0.14*      | 1       | 1     | 0.00  | 0.00  | 0.87   | 0.90  | 0.00  | 0.00  | 13.75 – | 15.62 – |
| F83      | 37.3             | 0.17       | 1       | 1     | 0.00  | 0.00  | 0.89   | 0.88  | 0.00  | 0.00  | 12.53 – | 12.60* – |
| F93      | 36.6             | 0.17       | 1       | 1     | 0.00  | 0.00  | 0.90   | 0.90  | 0.00  | 0.00  | 13.00 – | 12.68* – |
| F94      | 31.5*            | 0.15*      | 1       | 1     | 0.00  | 0.00  | 0.88   | 0.87  | 0.00  | 0.00  | 13.07 – | 14.28* – |
| Foc-W6-1(wt) | 40.2           | 0.19       | 1       | 1     | 0.00  | 0.00  | 0.86   | 0.90  | 0.00  | 0.00  | 12.93 – | 15.56 – |

*These strains were genetically transformed with the ZsGreen fluorescent protein according to Khang et al. (2006); (wt) indicates wild-type strains.

RMG = Radial Mycelial Growth Final, assessed by the average value of the fungal colony radius reached after 9 days growing at 25 ± 1 °C and a photoperiod of 12 h of a mixture of fluorescent white and near-UV light at 36 μE m⁻² s⁻¹. RGR = Radial Growth Rate: slope of linear regression model that related the temporal increase of the radial growth of the fungal colony. Each value is the mean of three replications (petri dishes). Means in a column followed by an asterisk are significantly different (P<0.05) than the mean for the corresponding wild-type isolate according to Dunnett’s test.

Disease was assessed on a 0 to 4 severity scale based on the percentage of affected foliar tissue (0 = 0%, 1 = 1 to 33%, 2 = 24 to 66%, 3 = 67 to 100%, and 4 = dead plant) at 2–3 days intervals for 42 days growing inside a walk-in growth chamber adjusted at 25±2 °C, 60–90% of relative humidity and a 14-h photoperiod of fluorescent white light at 360 μE m⁻² s⁻¹. Incidence and severity data were used to calculate a Disease Intensity Index (DII). DIIfinal = DII at the final date of disease assessment; SAUDIIPC = area under disease intensity index progress curve standardized by duration time in days of the epidemic; IP: Incubation period, estimated as the number of days to initial symptoms. Data are the means of four replicated pots, each with four plants. Means in a column followed by an asterisk are significantly different (P<0.05) than the mean for the corresponding wild-type isolate according to Dunnett’s test.

No disease symptoms observed.

*dNo disease symptoms observed.

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**Interaction** (Fig. 3i, Table 2). Two days later, the intercellular colonization of the root cortex was extensive in the susceptible cv. P-2245, and the pathogen reached the central root cylinder and entered into the xylem vessels of the upper root zone (Figs. 2j, 3c,d). Comparatively, the resistant reaction was characterized by restricted pathogen growth within the root tissues, but the degree of restriction varied between the two cultivars. By 8 dai the race 0 transformant remained restricted to intercellular spaces of the root cortex of ‘WR315’ (Fig. 2f) or could reach a few individual vessels in the lowermost root zone of ‘JG-62’ (Fig. 1k). Based on those observations, the root apex was identified as the preferential site of infection for the three chickpea cultivars by race 0, regardless their resistance (Fig. 2a–c).

By 10 dai, the race 0 transformant progressed upward to reach the hypocotyl, epicotyl, and lower stem zone of susceptible ‘P-2245’ seedlings. The first disease symptoms (i.e. yellowing of foliage at the stem base characteristic of the yellowing syndrome caused by race 0) was observed at 10 dai. Thereafter, the pathogen colonized the upper stem vascular tissue, reaching the fifth internode by 12 dai (Figs. 2m, 3e,f). By 16 dai, all ‘P-2245’ plants were severely affected, eventually resulting in plant senescence and decay. At this stage, fungal hyphae erupted from the stem vascular tissue into the surrounding cortical parenchyma.

In race 0-resistant ‘JG-62’ chickpea plants, the pathogen progressed upwards along the stem axis at a rate slower than that observed in the susceptible cv. P-2245. It took 12 dai for the pathogen to reach the lower stem xylem vessels (Fig. 2n). The pathogen grew up to reach the fourth stem internode, although no symptoms were observed during the entire duration of the experiment. Comparatively, in the race 0-resistant ‘WR-315’ plants, the pathogen remained restricted within the intercellular spaces of the cortex in the intermediate root zone, but it was never found in the vascular system (Fig. 2o). Infected plants remained free of symptoms throughout the duration of the experiment.

Early infection events of susceptible cvs. P-2245 and JG-62 by a transformant of the highly virulent *F. oxysporum* f. sp. *ciceris* race 5 were similar to those described for *F. oxysporum* f. sp. *ciceris* race 0 (Fig. 1, Table 2). From 1 to 4 dai, extensive growth of the fungus on the root surface and the root meristem was observed (Fig. 4a–c). Thereafter, growth of the race 5 transformant within plants of these two cultivars progressed faster compared with that of race 0 in cv. P-2245 (Fig. 4d–f). Colonization of xylem vessels in the apical root zone of cv. P-2245 by the race 5 transformant started as early as 6 dai and was preceded by extensive intercellular growth within the root cortex (Fig. 4g). Comparatively, no vascular infection was noticed in roots of cvs. WR-315 (incompatible interaction) and JG-62 (compatible interaction) at that same time, and the pathogen appeared restricted within the epidermis and root cortex of the apical root zone, respectively (Fig. 4h,i). However, by 8 dai hyphae of the race 5 transformant reached the stem xylem vessels and were located in the epicotyl and second stem internode of susceptible cvs. JG-62 (Fig. 4k) and P-2245 (Fig. 4j), respectively, but no symptoms were observed. Symptoms started to develop on day 9 dai, at which time the pathogen hyphae within the xylem vessels had reached up to the fourth and third stem internodes of ‘P-2245’ and ‘JG-62’ plants, respectively.
Symptoms on these two cultivars consisted of severe leaf chlorosis and flaccidity characteristic of the wilting syndrome. Growth of the pathogen within the stem xylem continued, subsequently reaching the uppermost stem tissues in ‘P-2245’, and the 3 to 4th internodes of ‘JG-62’ by 12 dai. Two days later, the entire vascular system was colonized by the pathogen, and the plants died. Conversely, plants of the resistant cv. WR-315 remained symptomless throughout the duration of the experiment, though race 5 was able to invade the xylem vessels at the apical root zone by 12 dai, and occasionally it was found in the hypocotyl xylem vessels by 18 dai. *F. oxysporum* f. sp. *ciceris* race 5 was found neither in epicotyl nor in stem tissues (Fig. 4o).

Colonization by the pathogen was quantitatively assessed using the incidence (proportion of colonized vessels, IcVC) and intensity (lumen area of the transverse section of an infected vessel occupied by fungal hyphae, ItVC) parameters. These two variables were highly and positively correlated ($r=0.7936$, $P<0.0001$); therefore, only results concerning data analysis of ItVC are shown. Overall, the ItVC was significantly influenced ($P<0.05$) by the experimental treatment combinations and their interactions. However, most ItVC variation was accounted by the main factors in the study, i.e., pathogenic race ($F=500.81$, $P<0.0001$, 46.16% Mean Square Error [MSE]) and chickpea cultivar ($F=279.16$, $P<0.0001$, 25.73% MSE). Variation in ItVC was accounted to a much lesser extent by sampling time ($F=75.13$, $P<0.0001$, 6.93% MSE) and

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Figure 2. Temporal and spatial patterns of chickpea infection by *Fusarium oxysporum f. sp. ciceris* race 0. Colonization of three cultivars, including P-2245 (susceptible, S), JG-62 (resistant, R), and WR-315 (R) was imaged using confocal laser scanning microscopy. ep: epidermis, c: cortex, x: xylem. dai: number of days after inoculation. (a–c): root apex; (d–i): lower root zone; (j): upper root zone; (k): intermediate root zone; (l): lower root zone; (m): stem fifth internode; (n): stem fourth internode; (o): half root zone. The *Fusarium oxysporum f. sp. ciceris* isolate was transformed with the ZsGreen fluorescent protein. doi:10.1371/journal.pone.0061360.g002

Figure 3. Advanced stages of chickpea infection by *Fusarium oxysporum f. sp. ciceris* races 0 and 5 in compatible and incompatible interactions. Left panels: cross sections; Right panels: longitudinal sections. (a,b) Intercellular colonization of cortical root tissue occurring in all compatible and incompatible interactions; (c–d) intercellular colonization of cortical root tissue and fungal mycelia through root xylem vessels occurring in all compatible interactions and in the incompatible interactions of cv. JG-62/race 0 and cv. WR-315/race 5; (e–f) heavy colonization of xylem stem vessels occurring in all compatible interactions and in the incompatible interaction of cv. JG-62/race 0. The *Fusarium oxysporum f. sp. ciceris* isolates were transformed with the ZsGreen fluorescent protein and imaged using confocal laser scanning microscopy. c: cortex, x: xylem. doi:10.1371/journal.pone.0061360.g003

variants in ItVC, which ranged from 5.59 to 0.67% of the MSE (data not shown).

At early stages of plant colonization, i.e., at sampling times in which no disease symptoms develop in the compatible interactions, ItCV was significantly higher (*F = 185.21, P < 0.0001*) on compatible interactions (Fig. 6a). Among them, ItVC was highest (2.81 ± 0.17%; *F = 169.04, P < 0.0001*) in the most conducive P-2245/race 5 interaction, decreased significantly (1.66 ± 0.10%; *F = 56.69, P < 0.0001*) in cv. JG-62 infected by this same race 5, and was lowest (0.51 ± 0.07%; *F = 168.03, P < 0.0001*) in plants of cv. P-2245 infected by race 0 (Fig. 6a). Among incompatible interactions, ItVC was highest on the cv. WR-315/race 5 interaction (1.17 ± 0.16%; *F = 65.18, P < 0.0001*) and decreased to 0.18 ± 0.04% (*F = 40.96, P < 0.0001*) in 'JG-62' plants infected by race 0. There was no invasion of xylem vessels in the cv. WR-315/race 0 interaction (Fig. 6a).

At advanced stages of plant colonization, i.e., in sampling times in which disease symptoms develop in compatible interactions, infection of xylem vessels only occurred in 'JG-62' plants infected by race 0 that showed a mean ItCV of 0.51 ± 0.08% (Fig. 6b). In compatible interactions, the mean ItVC was highest (*F = 310.85, P < 0.0001*) for the cv. P-2245/race 5 interaction with a mean ItVC of 5.87 ± 0.26%, followed by that in cv. JG-62 infected by the same race 5 (2.48 ± 0.15%; *F = 286.54, P < 0.0001*) (Fig. 6b). Comparatively, infection of those two cultivars by the less virulent race 0 was associated with significantly lower ItVC values, i.e., 1.65 ± 0.16% in cv. P-2245 (*F = 185.40, P < 0.0001*) and 0.51 ± 0.08% in the incompatible cv. JG-62/race 0 interaction (*F = 20.82, P < 0.0001*) (Fig. 6b). It should be emphasized that, as it was pointed out before, no symptoms developed in 'JG-62' infected by race 0.

Variation of mean ItVC along the main axis of a plant differed considerably among chickpea cultivar-pathogenic race experimental combinations, sampling times and the severity of symptoms (Fig. 5; Table 3). Thus, for asymptomatic plants in the incompatible cv. JG-62/race 0 interaction, the mean ItVC in root and hypocotyl was significantly higher (*F = 14.15, P = 0.0002*) than in epicotyl and stem (Fig. 5a–d), whereas the mean ItVC values in the epicotyl were similar to those in the stem (*F = 1.72, P = 0.1900*) (Fig. 5c,d; Table 3). Overall, the mean ItVC was similar (*F = 2.21, P > 0.1385*) in subterranean and aerial plant parts of the compatible cv. JG-62/race 5 interaction that were sampled at the same time that those in the incompatible cv. JG-62/race 0 interaction that remained asymptomatic (Fig. 5a–d). However,
ItVC in the epicotyl was significantly higher ($F=18.03, P<0.0001$) when compared with that in the stem (Fig. 5c,d; Table 3). Nevertheless, at later sampling times, with plants showing Fusarium wilt symptoms, the mean ItVC increased from bottom to top in a plant. Thus, the aerial plant tissues showed a higher colonization intensity ($F=6.43, P=0.0144$) compared with that in subterranean tissues, with ItVC being highest ($F<11.70, P<0.0007$) in the epicotyl (Fig. 5a–d; Table 3). However, this colonization pattern did not hold for plants sampled at 16 dai. At this time, severe symptoms in a plant were associated with extensive collapse and xylem degeneration, particularly in the aerial tissues, which determined the occurrence of ItVC means lower than those in previous sampling times (Fig. 5c,d).

In compatible interactions of the highly susceptible cv. P-2245 with the two races, the pattern of quantitative colonization during the time period that plants were asymptomatic was similar for races 0 and 5, with the mean ItVC being higher ($F=10.06, P<0.0017$) in the subterranean tissues compared to that in aerial tissues (Table 3), though mean values for race 5 were significantly higher than those for race 0 ($F=171.31, P<0.0001$) (Fig. 5e–h). Conversely, that pattern of colonization differed much between races when infection gave rise to the symptomatic stage (Fig. 5). Thus, for F. oxysporum f. sp. ciceris race 0 the ItVC was significantly higher ($F=44.53, P<0.0001$) in subterranean than in aerial plant parts (Fig. 5e–h; Table 3), and ItVC decreased in the upper stem tissues as the severity of symptoms increased ($F=51.61, P<0.0001$) (Fig. 5h; Table 3). On the contrary, for race 5 ItVC was higher ($F=39.71, P<0.0001$) in the aerial than in subterranean plant parts, reaching similar values ($F=2.83, P=0.0923$) in the epicotyl and upper stem tissues (Fig. 5e–h; Table 3).

**Discussion**

This study demonstrates for the first time the utility of CLSM for better understanding the spatial-temporal dynamics of the infection and colonization processes of F. oxysporum f. sp. ciceris races 0 and 5 in three chickpea cultivars including compatible and incompatible interactions.

The use of genetically transformed organisms requires prior demonstration that transformation did not modify any biological feature of the wild strains. Phenotypic characterization of the transformants developed in our study, including growth rate and morphology, pathogenicity and virulence on a susceptible chickpea cultivar, and pathogenic race, showed that most of them retained the biological features of the wild-type strain. Based on these evaluations, two transformants were selected for the study: F11 and F93 derived, respectively from wild strains Foc-7802 (race 0) and Foc-W6-1 (race 5).

We qualitatively and quantitatively assessed the interactions of F. oxysporum f. sp. ciceris races 0 and 5 with chickpea cultivars in both compatible and incompatible interactions from the early stages of root colonization to infection and extensive stem vascular colonization of three cultivars, showing differential resistance by *Fusarium oxysporum* f. sp. *ciceris* races 0 and 5*

**Table 2.** Colonization process of different parts of three chickpea cultivars showing differential resistance by *Fusarium oxysporum* f. sp. *ciceris* races 0 and 5.*

| Time (days after inoculation) | Cultivar | P-2245 | JG-62 | WR-315 |
|-----------------------------|----------|--------|--------|--------|
| *F. oxysporum* f. *sp. ciceris* race 0 | | | | |
| 1 to 3 Root (L): epidermis | Root (L): epidermis | Root (L): epidermis |
| 4 Root (L): epidermis | Root (L): epidermis | Root (L): epidermis |
| 6 Root (L): cortex | Root (L): cortex | Root (L): epidermis |
| 8 Root (U): xylem vessels | Root (L): xylem vessels | Root (L): cortex |
| 10 Stem (L): xylem vessels | Root (U): xylem vessels | Root (M): cortex |
| 12 Stem (M): xylem vessels | Stem (M): xylem vessels | Root (M): cortex |
| 14 Stem (U): xylem vessels | Stem (M): xylem vessels | Root (M): cortex |
| *F. oxysporum* f. *sp. ciceris* race 5 | | | | |
| 1 to 3 Root (L): epidermis | Root (L): epidermis | Root (L): epidermis |
| 4 Root (L): cortex | Root (L): cortex | Root (L): epidermis |
| 6 Root (L): xylem vessels | Root (M): cortex | Root (L): epidermis |
| 8 Stem (L): xylem vessels | Epicotyl: xylem vessels | Root (L): cortex |
| 10 Stem (M): xylem vessels | Stem (L): xylem vessels | Root (L): cortex |
| 12 Stem (U): xylem vessels | Stem (M): xylem vessels | Root (L): xylem vessels |
| 14 Stem (U): xylem vessels | Stem (U): xylem vessels | Root (L): xylem vessels |

*Progress of pathogen proliferation along the vertical axis of the plant was analyzed in the following parts: Root: (L) lower: meristem, elongation and differentiation zones; (M) medium, (U) upper. Stem: (L) lower, internode 1 to 2nd; (M) medium, internode 3 to 4th; (U) upper, 5th internode or above.

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Figure 5. Intensity of vascular colonization (ItCV) by races 0 and 5 of *Fusarium oxysporum* f. sp. *ciceris*. The *Fusarium oxysporum* f. sp. *ciceris* (Foc) isolates were transformed with the ZsGreen fluorescent protein. Plants were sampled daily from 1 to 4 days after inoculation (dai), and at 2 days intervals from 6 to 18 (dai). Tissues sampled included: Root: lower and intermediate zone of the tap root (a, e); Hypocotyl (b, f) and epicotyl (c, g): zones immediately before and after the insertion of cotyledons, respectively; Stem (d, h): internodes one to fifth of the main stem. Severity (S)
indicate the disease severity (DS) level of foliar symptoms assessed at each sampling time using a 0 to 4 scale based on the percentage of affected foliar tissue (0 = 0%, 1 = 1 to 33%, 2 = 24 to 66%, 3 = 67 to 100%, and 4 = dead plant). S = 0: no symptoms; S = L: initial symptoms (0 < DS < 2); S = M: moderate symptoms (2 < DS < 3); and S = S: severe symptoms (DS ≥ 3), for Foc-0 (first character) and Foc-5 (second character). There were four plants (two plastic pots) per F. oxysporum f. sp. ciceris race/chickpea cultivar combination analysed for each sampling time. The experiments were arranged in a randomized complete blocks design, and were repeated twice. For each sampled tissue, vascular colonization was assessed on four blocks with 20 cells (xylem vessels) per block, covering the entire vascular cylinder. Error bars indicate the standard error of the mean. Significant differences (P < 0.05) between ItCV level reached on races 0 and 5 for each chickpea cultivar/tissue combination at each sampling date are indicated by an asterisk.

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Figure 6. Intensity of vascular colonization (ItCV) by races 0 and 5 of Fusarium oxysporum f. sp. ciceris at early and advanced stages of plant colonization. (a) ItCV mean values at early stages of plant colonization, i.e., sampling times in which no disease symptoms develop in the compatible interactions. (b) ItCV mean values at advanced stages of plant colonization, i.e., at sampling times in which symptoms develop in compatible interactions. The Fusarium oxysporum f. sp. ciceris (Foc) isolates were transformed with the ZsGreen fluorescent protein. I: incompatible interaction; C: compatible interaction. Plants were sampled daily from 1 to 4 days after inoculation (dai), and at 2 days intervals from 6 to 18 (dai). Tissues sampled included: Root: lower and intermediate zone of the tap root; Hypocotyl and epicotyl: zones immediately before and after the insertion of cotyledons, respectively; Stem: internodes one to fifth of the main stem. There were four plants (two plants per pot) per F. oxysporum f. sp. ciceris race/chickpea cultivar combination analysed for each sampling time. The experiments were arranged in a randomized complete blocks design, and were repeated twice. For each sampled tissue, vascular colonization was assessed on four blocks with 20 cells (xylem vessels) per block, covering the entire vascular cylinder. Error bars indicate the standard error of the mean. Significant differences (P < 0.05) between ItCV level reached at racers 0 and 5 for each chickpea cultivar are indicated by an asterisk.

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colonization. In contrast to our work, most studies on formae specialiae of F. oxysporum/host plant interactions involving the use of CLSM were restricted to subterranean host tissues [e.g., 18,19,33,34,35,36,37], with only a few of them trying to observe colonization in the stem tissues [20,21,30].

Ingress of formae specialiae of F. oxysporum into host plants was assumed to take place either by direct penetration or through wounds [39]. In this present study, penetration of chickpea roots by F. oxysporum f. sp. ciceris races 0 and 5 occurred through intact tissues without need of differentiating specialized penetration structures. This confirms previous observations in this same pathosystem by Jiménez-Diaz et al. [23], as well as most works performed in other Fusarium wilts [e.g., 17,18,21,39,40,41,42,43]. In our study, the meristematic cells of chickpea root apex were identified as the preferential infection site for F. oxysporum f. sp. ciceris. This does not agree with previous studies on this pathosystem, which concluded that the cotyledons and zones of the epicotyl and hypocotyl at the junction of or close to cotyledons are preferential invasion sites of the pathogen [23,24]. Differences in the inoculation methods used may account for differences between these studies. Indeed, Olivain & Alabouvette [43] observed that colonization of the root elongation zone and root apex were the preferential infection sites of tomato roots inoculated with F. oxysporum f. sp. lycopersici by the hydroponic culture method, but that did not hold when plants were inoculated by the soil-infestation inoculation method [22,35].

In the absence of wounds, most formae specialiae of F. oxysporum can get ingress into the host through a variety of root tissues, including the root meristem, root hairs, and different zones of the root and seed [39]; however, most reports support the root tip as a preferential site for host invasion by these pathogens. This was reported for F. oxysporum f. sp. asparagi in asparagus [44], F. oxysporum f. sp. dianthi in carnation [45], F. oxysporum f. sp. lini in flax [46], F. oxysporum f. sp. pisici in pine seedlings [47], F. oxysporum f. sp. vanillae in vanilla [48], and F. oxysporum f. sp. vasarum in cotton [42]. The lack of penetration through the root host apex was described only for F. oxysporum f. sp. cucumerinum in cucumber [49]. Others reported that host penetration sites for formae specialiae of F. oxysporum include the zone of root elongation by F. oxysporum f. sp. lentis in lentil [50], and the cotyledonal node and undifferentiated region of the root tip for F. oxysporum f. sp. pisici in peas [51]. In our study, the rate and intensity at which the pathogen colonized the epicotyl and stem xylem was directly related to the degree of compatibility of the F. oxysporum f. sp. ciceris race/chickpea cultivar combination. Thus, both in the root and stem the fastest and higher intensity of xylem colonization (ItVC) occurred in the compatible interactions. Thus, ItCV was highest in cv. P-2245 infected by the highly virulent race 5, followed by that in cv. JG-62 infected by the same race and in cv. P-2245 infected by the less virulent race 0. This quantitative pattern of xylem colonization agrees with observations by Garcia-Sánchez et al. [20] in bean infected with F. oxysporum f. sp. phaseoli, in which the rate and intensity of colonization of xylem vessels were higher for the most virulent isolate of the pathogen. The higher biomass of race 5 compared with that of race 0 observed and quantified with CLSM
confirms results from our previous studies using real-time quantitative PCR, in which the quantity of race 5 DNA both in subterranean and aerial plant tissues was significantly higher than that of race 0 [52].

Results of particular interest from this present study are those concerning the incompatible interactions. In general, asymptomatic reactions in fungal wilt pathosystems are associated with the absence of vascular invasion by wilting fungi. In our study, that occurred only for the cv. WR-315/race 0 combination. However, when this same cultivar was infected by the more virulent race 5, the pathogen either invaded the root xylem vessels to a limited degree or extensively colonized the root and stem xylem vessels, respectively, but without any symptom development. This observed lack of vascular colonization in highly incompatible chickpea/F. oxysporum f. sp. ciceris interactions agrees with reports from other authors that used light microscopy for observations of paraffin-embedded tissues [23,24]. Stevenson et al. [24] failed to find the fungus in the xylem vessels of any of the resistant cvs. WR-315 and CIPS-1 inoculated with each of races 1A or 2, and Jiménez-Díaz et al. [23] reported the absence of fungal structures in root and stem tissues of resistant ‘WR-315’ inoculated with either race 0 or 5, and resistant ‘JG-62’ inoculated with race 0. However, these later authors also reported positive isolation of the pathogen in artificial growth media from subterranean tissues located at or near sites of cotyledon attachment. From this, Jiménez-Díaz et al. [23] concluded that F. oxysporum f. sp. ciceris can remain associated with those tissues from which it could be isolated, though to such a minor extent that no fungal structures could be observed microscopically. Our results for the incompatible cv. WR-315/race 5 interaction are consistent with the observations by those authors and demonstrate the high efficiency of the CLSM technology to visualize and characterize in vivo host-pathogen interactions.

The difference in the degree of colonization of resistant cultivars by F. oxysporum f. sp. ciceris races found in this present study was also reported for other formae speciales of F. oxysporum in resistant cultivars of different hosts. Fang et al. [38] indicated that F. oxysporum f. sp. fragariae remained confined in the epidermal layer of the root in a resistant strawberry cultivar, and Tessier et al. [53] described that F. oxysporum f. sp. pipi was restricted to the initially infected root vessels in asymptomatic reactions of a resistant pea cultivar. Conversely, Zvirin et al. [21] reported vascular colonization of a resistant melon cultivar by F. oxysporum f. sp. melonis race 1.2, although that occurred at a lower rate and incidence than that in the susceptible cultivar. Similarly, Ramírez-Suero et al. [19] indicated that there were no significant differences between the degree of colonization of susceptible and tolerant M. truncatula genotypes by F. oxysporum f. sp. medicaginis.

Less common among Fusarium wilt diseases is the occurrence of extensive vascular colonization of the plant axis in the absence of visible symptoms, as observed in this study for cv. JG-62 infected with race 0. On the contrary, the degree of symptom expression is usually reported as being closely related to the extent of vascular colonization by the pathogen [54], as it was found in this compatible chickpea/F. oxysporum f. sp. ciceris interactions in this present study. Interestingly, cv. JG-62 is susceptible to all known races of the pathogen causing the wilting syndrome but shows a moderate reaction to race 1B/C, which together with race 0, belongs to the yellowing pathotype [55]. The asymptomatic response of ‘JG-62’ plants to extensively xylem colonization by race 0 corresponds to a tolerant reaction rather than true

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Table 3. Effects of sampling time and plant tissue on vascular colonization intensity by Fusarium oxysporum f. sp. ciceris (Foc) races 0 and 5 in chickpea cultivars showing differential resistance.

| Source of variation a | Cultivar JG-62 | Cv. P-2245 |
|-----------------------|----------------|------------|
|                       | Foc-0          | Foc-5      | Foc-0          | Foc-5      |
| Sampling time         |                |            |                |            |
| Plant zone            |                |            |                |            |
| Root vs. Hypocotyl    | 14.15          | 0.0002     | 6.77           | 0.0002     |
| Hypocotyl vs. Epicotyl|                |            | -              |            |
| Epicotyl vs. Stem     |                |            | 1.72           | 0.0002     |

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**Notes:**
- "Plants were grown hydroponically in a growth chamber at 25 ± 2°C and a 14-h photoperiod of fluorescent white light of 360 μE m⁻² s⁻¹ in an orbital shaker at 120 rpm. Plants were sampled at 2 days intervals from 6 to 18 days after inoculation (dai). Tissues sampled included: Root: lower and intermediate zone of the tap root; Hypocotyl vs. Epicotyl – – 18.05 0.0001 4.58 0.0328 0.93 0.3347 1.20 0.0001 37.00 0.0001 11.56 0.0007 0.0001 20.77 0.0001 6.47 0.0001
- "Linear single-degree-of-freedom contrasts were computed to test the effect of selected experimental treatment combinations at P = 0.05.
- "Experimental combinations in which no disease symptoms developed.
- "Underlined values indicate that estimated value for the first term of the contrast was lower than that of the second term.

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resistance. This could indicate that either the fungal biomass has not yet reached the threshold for inducing symptoms or, more likely, that other mechanisms together with the presence of pathogen in the vascular tissues are required for the disease to develop.

The differential patterns of infection and colonization of chickpea cultivars by different races could be related to differences in the defence mechanisms between resistant and susceptible cultivars. In fact, Garcia-Limones et al. [56] indicated that infection by F. oxysporum f. sp. ciceris race 5 caused significant changes in activity levels of several oxidative stress-related enzymes in the apoplast of chickpea roots, although there were differences between susceptible (‘JG-62’) and resistant (‘WR-315’) cultivars. Thus, while infection by the pathogen increased apoplastic glutathione reductase (GR) activity and decreased superoxide dismutase (SOD) and diamine oxidase (DAO) activities in the susceptible and resistant cultivars, that occurred earlier (i.e., GR and SOD) or in higher quantities (i.e., DAO) in the resistant (‘WR-315’) than in the susceptible (‘JG-62’) cultivar. Conversely, the main specific responses to infection in each interaction were an increase in apoplastic ascorbate peroxidase activity in cv. WR-315 and a decrease of this activity in cv. JG-62.

Finally, the results in our study are relevant for disease control. It is well accepted that the use of resistant cultivars is one of the few and most effective and environmentally friendly control measures for Fusarium wilt of chickpeas [4,57,58]. However, populations of F. oxysporum f. sp. ciceris display high pathogenic variability, which limits the effectiveness and extensive use of available resistance and makes necessary the adequate characterization of resistance of chickpea lines and cultivars to specific races of F. oxysporum f. sp. ciceris [52,57,59,60]. Our work demonstrates the utility of CLSM as a rapid and efficient method to qualify and quantify infection by F. oxysporum f. sp. ciceris races in plant roots and stem, which allows for the accurate assessment of disease reactions of chickpea germplasm particularly in incompatible interactions. This technique allows selecting breeding lines with complete resistance or those that being partially resistant may carry traits of agronomic and/or commercial interest. Thus, alone or in combination with the q-PCR protocol developed in a previous study [52], use of CLSM is a powerful tool for studies aimed at gaining better understanding of chickpea - F. oxysporum f. sp. ciceris interactions and the efficient management through disease resistance breeding in Fusarium wilt of chickpeas.

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Author Contributions

Conceived and designed the experiments: DJ-F BL RMJ-D JN-C. Performed the experiments: DJ-F BL SK JN-C. Analyzed the data: DJ-F BL JN-C. Contributed reagents/materials/analysis tools: DJ-F BL SK RMJ-D JN-C. Wrote the paper: DJ-F BL SK RMJ-D JN-C.

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