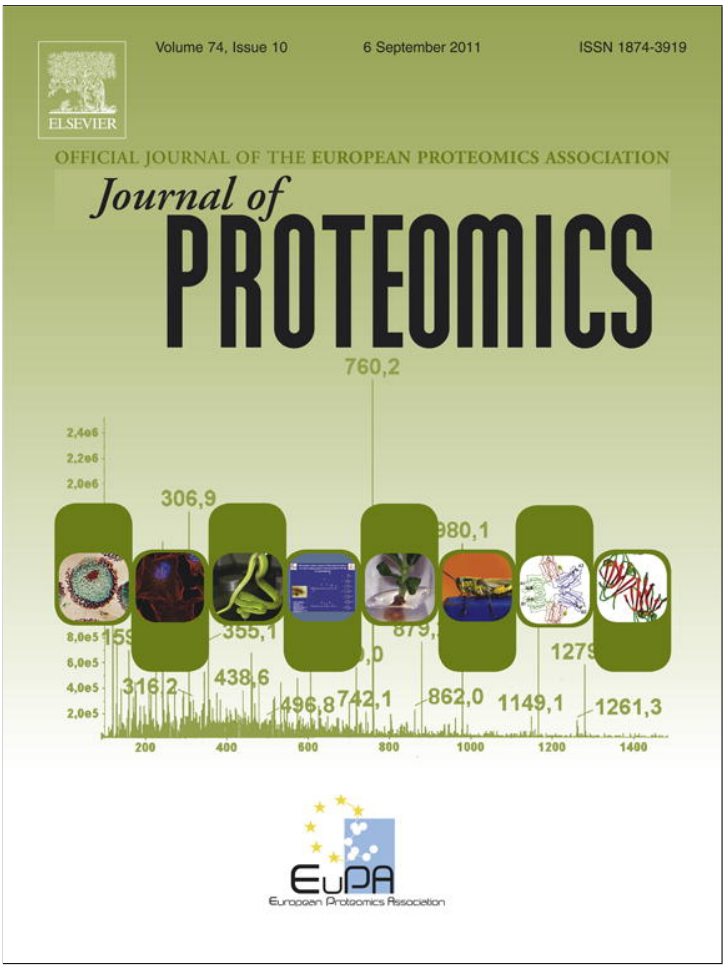

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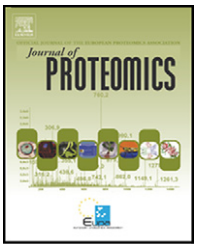

A proteomic study of in-root interactions between chickpea pathogens: The root-knot nematode *Meloidogyne artiellia* and the soil-borne fungus *Fusarium oxysporum* f. sp. *ciceris* race 5

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ABSTRACT

Fusarium wilt caused by the fungus *Fusarium oxysporum* f. sp. *ciceris* (Foc) is the main soil-borne disease limiting chickpea production. Management of this disease is achieved mainly by the use of resistant cultivars. However, co-infection of a Foc-resistant plant by the fungus and the root-knot nematode *Meloidogyne artiellia* (Ma) causes breakdown of the resistance and thus limits its efficacy in the control of Fusarium wilt. In this work we aimed to reveal key aspects of chickpea:Foc:Ma interactions, studying fungal- and nematode-induced changes in root proteins, using chickpea lines 'CA 336.14.3.0' and 'ICC 14216 K' that show similar resistant (Foc race 5) and susceptible (Ma) responses to either pathogen alone but a differential response after co-infection with both pathogens. 'CA 336.14.3.0' and 'ICC 14216 K' chickpea plants were challenged with Foc race 5 and Ma, either in single or in combined inoculations, and the root proteomes were analyzed by two-dimensional gel electrophoresis using three biological replicates. Pairwise comparisons of treatments indicated that 47 protein spots in 'CA 336.14.3.0' and 31 protein spots in 'ICC 14216 K' underwent significant changes in intensity. The responsive protein spots tentatively identified by MALDI TOF-TOF MS (27 spots for 'CA 336.14.3.0' and 15 spots for 'ICC 14216 K') indicated that same biological functions were involved in the responses of either chickpea line to Foc race 5 and Ma, although common as well as line-specific responsive proteins were found within the different biological functions. To the best of our knowledge, this is the first study at the root proteome level of chickpea response to a biotic stress imposed by single and joint infections by two major soil-borne pathogens.

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1. Introduction

Chickpea (*Cicer arietinum* L.) is one of the most important cool-season food legumes grown worldwide and is an important protein source in many semi-arid tropic regions [1]. However, chickpea production is limited by Fusarium wilt and root-knot nematode infections, particularly in the Mediterranean Basin and Indian subcontinent [2].

Root-knot nematodes of the genus *Meloidogyne* encompass more than 90 nominal species distributed worldwide and are among the most successful plant parasites [3]. Parasitism by root-knot nematodes is characterized by the establishment of permanent feeding sites comprised of multinucleate giant cells in the root cortex, endodermis, pericycle, and vascular parenchyma of host plants. The feeding sites act as sinks for plant photosynthates and impair plant growth and development. In addition, deformation and blockage of vascular tissues at feeding sites limits translocation of water and nutrients in the plant, further suppressing plant growth and crop yield. Tissues surrounding the feeding sites of root-knot nematodes usually swell, giving rise to large, characteristic galls on the roots of infected plants. However, infection of chickpea roots by *M. artiellia* (*Ma*) only gives rise to very small galls surrounding the feeding sites [4].

Fusarium wilt of chickpea, caused by the fungus *Fusarium oxysporum* f. sp. *ciceris* (*Foc*), is the most important soil-borne disease limiting chickpea production worldwide [5]. Management of this disease is achieved mainly by the use of resistant cultivars. However, the efficacy of resistant cultivars can be curtailed by the existence of pathogenic races in *Foc* populations and by co-infection of resistant plants by the fungus and plant-parasitic nematodes [6]. In particular, co-infections with *Foc* and *Meloidogyne* spp. can lead to breakdown of resistance against certain races of the fungus [6–8]. It has recently been shown that infection of chickpea lines partially resistant to the highly virulent *Foc* race 5 by *Ma* invariably increased their susceptibility to Fusarium wilt, whereas infection of lines with complete resistance to that race of the fungus led to loss of Fusarium wilt resistance in certain lines but did not affect the resistance phenotype in others [6].

Proteomics has the potential to explain plant–pathogen interactions because proteins act as the immediate perpetrators of biological actions or responses. Proteomic analysis of plants subjected to contrasting stressful treatments represents a powerful approach to identify responsive proteins that may be involved in mechanisms of stress susceptibility or tolerance. However, studies of this kind have mostly been conducted with plant model species for which the complete genome sequences are known (such as *Arabidopsis* or rice), and in relation to responses to abiotic rather than biotic stresses [9]. Furthermore, the relatively small number of proteomic studies on plant–pathogen interactions have mainly been carried out on bacterial and fungal diseases of foliar tissues as compared with root diseases caused by soil-borne fungi and nematodes. These studies have focused mainly on relatively simple two component plant–pathogen systems rather than on tripartite interactions between a plant and two pathogens. Recent reviews have emphasized the difficulty in carrying out proteomic studies on interactions between root-pathogens and their hosts [10]. Consequently there are few studies on proteomic responses of plants to nematode infection [11] or on systems involving plants and two or more microorganisms [12]. There are very few examples of proteomic studies that cover the organisms involved in the tripartite system in our study. The chickpea response to dehydration stress was analyzed at the proteome level in studies involving nuclear and extracellular matrix subproteomes from seedlings, but this work did not include roots [13,14]. To the best of our knowledge, no study has explored the chickpea root proteome or its response to any kind of pathogen. Regarding Fusarium wilt diseases, proteomic studies include analyses of the xylem sap subproteome in the *F. oxysporum* f. sp. *lycopersici*/tomato pathosystem [15], and of the global root proteome in the *F. oxysporum* f. sp. *betae*/sugar beet pathosystem [16]. Finally, there is one, unique study on root proteomics during nematode infection that concerns the interactions of resistant coffee and cotton genotypes with *Meloidogyne paranaensis* and *M. incognita*[17].

This present study aimed to examine root proteomic responses associated with the tripartite interaction of chickpea with two major soil-borne pathogens, *Ma* and *Foc* race 5 (*Foc*-5). Chickpea lines CA 336.14.3.0 and ICC 14216 K, which have similar susceptible (to *Ma*) or resistant (to *Foc*-5) responses to either pathogen alone, were used for this study. However, the resistance to *Foc*-5 is overcome (CA 336.14.3.0) or not (ICC 14216 K) after co-infection with *Foc*-5 and *Ma*. Therefore, 2-DE protein gels root tissues of the two chickpea lines were compared after inoculation with one or both pathogens. Spots showing presence/absence or changes in the amount between lines or treatments were trypsin-digested and subjected to MALDI-TOF/TOF for further identification. The results are discussed in terms of the possible functional implications of proteins identified, with special emphasis on their putative roles in the plant defense against pathogen infection. To our knowledge, this is the first study describing the root proteome response of chickpea to plant pathogens as well as the second study describing the plant-root proteome response to parasitic root-knot nematodes.

2. Materials and methods

2.1. Biological material and experimental design

A *Ma* isolate from chickpea roots in Castel del Monte (Bari province, southern Italy) and Foc 8012 monoconidial isolate of *Foc*-5 from chickpea in southern Spain [18] were used in this study. Nematode inoculum was increased from a single egg mass on chickpea cv. UC27. Nematode eggs were extracted by the NaOCl procedure [19]. The fungal inoculum consisted of chlamydospores produced as described previously [6].

Chickpea lines CA 336.14.3.0 and ICC 14216 K were used. 'CA336.14.3.0' is an advanced F_7 line with complete resistance to *Foc* races 5, 0, 1A and 6 derived from chickpea line ICCL 810001 [6]. 'ICC 14216 K' is of Mexican origin and deposited at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT). This line is resistant to *Foc* races 5, 0, 1A and 2 [20]. Both chickpea lines are similarly susceptible to *Ma*. However, the two lines respond differently to co-infection

with *Ma* and *Foc*-5 at inoculum densities of 3,000 and 30,000 chlamydospores/g soil. Under these conditions resistance to *Foc*-5 is overcome in CA 336.14.3.0 but not in ICC 14216 K [6].

Experiments were conducted in a growth chamber set to 25±1 °C, 60 to 90% relative humidity, and a 14-h photoperiod of fluorescent light of 360±25 μE m^{-2} s^{-1}. These environmental conditions are optimal for the development of Fusarium wilt in chickpea [18] and favorable for the reproduction and infection by *Ma*[6]. Chickpea seeds were surface-sterilized with 2% NaOCl for 3 min and germinated on sterile, moistened filter paper in Petri plates at 25±1 °C in darkness for 48 to 72 h. Germinated seeds, selected for uniformity (1 to 2 cm root length), were sown in 15-cm-diameter clay pots (four plants per pot) filled with the autoclaved soil mixture infested with 0 or 30,000 chlamydospores of *Foc*-5/g of soil and with 0 or 20 eggs and J2s of *M. artiellia*/cm^3 of soil. The nematode inoculum for each pot was suspended in 5 ml of sterile distilled water and added onto the chickpea seeds at the time of sowing. Sterile distilled water (5 ml) was added in treatments without the nematode. The inoculum density of *Foc*-5 in the infested soil was determined by dilution plating on V8 juice-oxgal-PCNB agar (VOPA) Fusarium-selective medium just before sowing. The potted plants were watered as needed and fertilized with 100 ml of a 0.1% solution of a 20–5–32+micronutrients hydro-sol fertilizer (Haifa Chemicals, Haifa, Israel) every week. The experiment consisted of a factorial treatment design comprising four treatments and consisted of uninoculated control plants (C treatment), plants inoculated only with either *Foc*-5 (F treatment) or *Ma* (M treatment), and plants co-inoculated with both *Foc*-5 and *Ma* (FM treatment) and it was repeated three times. Root samples were harvested at 35 to 40 days after inoculation, at which time high large numbers of *Ma* giant cells are formed without severe impairment of susceptible chickpeas [4]. Root samples consisted of 100 mg of galled root sections (for treatments involving *Ma* and plants inoculated with both pathogens) or equivalent root pieces (for un-inoculated and *Foc*-5 inoculated plants) (Fig. 1) harvested from the four plants in a pot. Soil from plants was removed under sterile tap water and roots were placed in sterile tap water in a glass Petri dish for further manipulations. Nematode female bodies and egg-masses were carefully removed from the sampled galls to minimize cross-contamination of the sampled root tissues with proteins of nematode origin. Root samples were placed in a microcentrifuge tube, water was removed and the root samples were frozen in liquid nitrogen.

2.2. Protein extraction and 2-DE

Root tissues were ground to a fine powder in a precooled mortar with liquid nitrogen. The powder was suspended in 10 mL of 0.5 M Tris–HCl, pH 7.5 lysis buffer containing 0.7 M sucrose, 50 mM EDTA, 0.1 M KCl, 10 mM thiourea, 2 mM PMSF and 2% v/v β-mercaptoethanol. The suspension was mixed with 10 mL of buffered phenol (pH 8) for 30 min and the phenolic phase, separated by centrifugation at 10,000 *g*, was rinsed with 10 mL of lysis buffer. Proteins were precipitated overnight at −20 °C after addition of five volumes of methanol containing 0.1 M ammonium acetate and pellets were recovered by centrifugation, rinsed with cooled methanol and acetone, dried under a stream of filtered air and resuspended in 200 μl of solubilisation buffer containing 9 M urea, 4% w/v CHAPS, 0.5% v/v Triton X-100, 100 mM DTT and 2% v/v biolytes pH 3–10 (Bio-Rad, USA). Insoluble components were removed by centrifugation (15,000 *g*, 15 min) and the supernatant protein content was quantified by the Bradford method using ovalbumin as a standard. Solubilized proteins were stored at −80 °C until use. For 2-DE analysis of protein extracts, the first IEF dimension was carried out in 17 cm immobilized pH gradient strips, pH 3 to 10 non-linear (Bio-Rad), which were rehydrated with 100 μg of protein in a final volume of 300 μl rehydration solution containing 9 M urea, 4% w/v CHAPS, 0.5% v/v Triton X-100 and 100 mM DTT. Focusing was carried out in a PROTEAN IEF Cell (Bio-Rad) and equilibration was performed following manufacturer's recommendations (Bio-Rad) (see Appendix S1 for details). For the second SDS-PAGE dimension, the IPG strips were immediately equilibrated according to Gómez-Vidal et al. [21] and placed on top of vertical 11% SDS-polyacrylamide gel slabs. Electrophoresis was run at 50 mA/gel using the PROTEAN Plus Dodeca Cell system (Bio-Rad) until the dye front reached the bottom of the gel. Gels were stained with Sypro Ruby Protein Gel Stain (Bio-Rad) according to the manufacturer's recommendations. Gel images were captured with FX-ProPlus (532 nm) MAC (Bio-Rad) and analyzed with PD-Quest™ software (Bio-Rad) using tenfold over background as a minimum criterion for presence/absence. The analysis was re-evaluated by visual inspection, focusing on the spots more drastically altered (close to two time expression at least in some of the treatments) among treatments and/or chickpea lines and that were consistently represented across technical replicates. All treatments, with their replicates for each chickpea line, were analyzed simultaneously. Normalized optical spot densities (individual spot intensity/normalization factor, calculated for each gel based on total quantity in valid spots) were quantified for each differential protein spot selected.

2.3. MS analysis and protein identification

Identification of proteins was accomplished using mass spectrometry (see Appendix S1 for detailed description). The excision of the selected spots from the gels was performed mechanically using a station Investigator ProPic (Genomic Solutions, USA). Gel fragments were digested with trypsin in a ProGest digestion station (Genomic Solutions). Peptide mass fingerprints (PMFs) were analyzed in the m/z range from 800 to 4000 Da by mass spectrometry MALDI-TOF/TOF, using a mass spectrometer 4700 Proteomics Analyzer (Applied Biosystems, USA) in automatic mode. The spectrum internal calibration was made using the porcine trypsin autolysis peptides ($M+H^+$=842.509, $M+H^+$=2211.104), with an average accuracy of ± 20 ppm. From each sample the fragmentation spectrum (MS/MS) was obtained from the three most intense m/z values.

Protein identification was carried out combining PMF and MS/MS data, which were used to search against MSDB database using the MASCOT search engine program (MatrixScience, UK), limiting the taxonomy category to plants. In addition, spots absent in control gels were also checked in the global database to detect putative contaminant proteins of nematode or fungal origin. Additional parameters used for

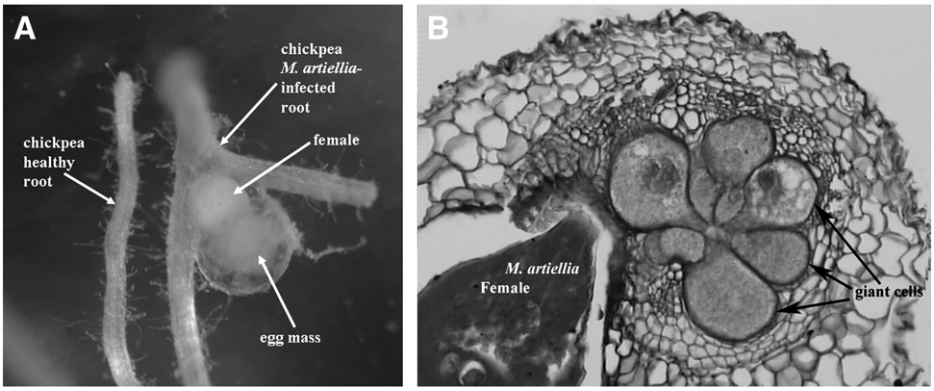


Fig. 1 – Healthy and *Meloidogyne artiellia*-infected chickpea roots, showing the prominent adult female covered by the egg mass (A); and cross-section of *M. artiellia*-infected root showing the typical feeding site with giant cells (B).

database searches were complete carbamidomethylation of cysteine residues and partial oxidation of methionine residues, peptide mass tolerance of 100 ppm, and one cutting mistake allowed. Parameters for the robustness of the MS identification were as follows: (i) protein score (more than 70) and protein score CI% (close to 100), (ii) peptide count, (iii) total ion score and total ion score CI% (close to 100%), and (iv) species matched (legume or related species).

2.4. *Statistical analysis of data*

The amount of proteins present in various spots of interest were analyzed using the web-based NIA array analysis tool ([22], available at http://lgsun.grc.nia.nih.gov/anova). Analyses were done in three phases: 1) hierarchical clustering of experimental conditions and repetitions for determining similarity between them; 2) principal component analysis (PCA) for identifying patterns of protein levels patterns in the set of responsive proteins; and 3) determination of the differences in levels of responsive proteins by pairwise comparisons of treatments included in the experiment (namely comparisons: F/C, M/C, FM/C, M/F, FM/F and FM/M). For these analyses the following settings were used: maximum of averaged and actual error variance as error model; 0.01 proportion of highest variance values to be removed before variance averaging; 10 degrees of freedom for the Bayesian error model; 0.05 FDR (False Discovery Ratio) threshold; and zero permutations. Hierarchical clustering analysis was done using the average distance method. PCA analyses were done using the Singular Value Decomposition (SVD) method using the covariance matrix, selecting three PCAs, one-fold change threshold for clusters and 0.4 correlation threshold for clusters. For pairwise comparisons of the amount of protein present, mean values of selected treatments, a 0.05 FDR, and one-fold threshold were used.

The constitutive spots data from the chickpea lines were subjected to analysis of variance (ANOVA) and LSD all-pairwise comparison test using Statistix 9.0 (NH Analytical Software, Roseville, MN, USA). Before analysis, raw data were normalized by transforming them to $log10(X)$ to detect statistical differences between the quantification of the same spot in all replicates and treatments [23].

3. Results and discussion

3.1. *Chickpea proteome analysis*

Preliminary proteome analyses of the complete root system of 'CA 336.14.3.0' and 'ICC 14216 K' seedlings infected with *Foc*-5 and *Ma* sampled 4 and 8 days after inoculation revealed very few proteome changes. This is likely to be due to a strong response dilution in the sampled tissues (data not shown). Conversely, use of infection-localized root tissues sampled 35 to 40 days after inoculation proved useful for the study. Approximately 110–200 μg of proteins were obtained from 100 mg of tissue. Fig. 2 shows a representative 2-DE gel corresponding to one of the treatments ('ICC 14216 K'–*Ma* interaction) in the study, in which a very good resolution of protein spots was obtained for the 3–10 (non-linear) p*I* and 20–120 kDa Mr range. About 350 protein spots were found to be consistently present throughout all 2-DE gels in the study (Fig. 2).

Differentially-expressed proteins were selected based on both visual inspection of 2-DE gels and quantitative data provided by the PDQuest analysis. These differences represented quantitative or, less frequently, qualitative changes (presence/absence) in protein spot intensity in the different treatments. Initially, comparisons of inoculated *vs.* control root samples allowed 64 protein spots to be selected for further analysis. These proteins were potentially present at different levels in the different samples. This number of potentially responsive proteins included 18 common protein spots to both chickpea lines, together with 33 and 13 specific protein spots from 'CA 336.14.3.0' and 'ICC 14216 K', respectively (Fig. 3A–B). Comparisons between the two uninoculated (control) chickpea lines showed similar root proteomes considering proteins well represented trough all gel replicates. Only two qualitative differences (one spot exclusively present in 'ICC 14216 K' and one spot specific to 'CA 336.14.3.0') and two quantitative differences (two spots with higher levels of protein present in 'ICC 14216 K' than in 'CA 336.14.3.0') were found (Fig. 3C).

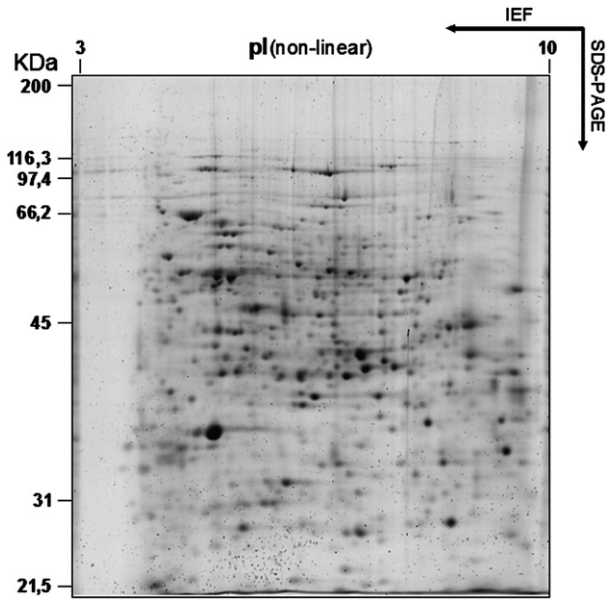


Fig. 2 – 2-DE protein profile of Sypro Ruby-stained root proteins from chickpea (*Cicer arietinum* L.) line ICC14216K inoculated with *Meloidogyne artiellia* at 35–40 DAI (Days After Inoculation). 100 μg of proteins were loaded and resolved on first-dimension (pH 3–10 non-linear gradient) and second dimension, SDS-PAGE on an 11% acrylamide gel. The gel corresponds to galled root extracts from secondary roots. Molecular mass is given on the left, while pI is given at the top of the figure.

3.2. *Changes in protein levels in response to pathogen infection*

Hierarchical clustering using the 18 protein spots affected in both chickpea lines indicated similar level profiles regardless of the inoculation treatment, with the exception of the *Foc*-5 (F)+*Ma* (M) treatment. Thus, the response of 'CA 336.14.3.0' to the FM treatment (which overcomes resistance to *Foc*-5) was located in a cluster separated from the FM treatment in 'ICC 14216 K' (which resistance to *Foc*-5 is not overcome) (Fig. 4A), jointly with the response of the two lines to the M treatment (Fig. 4A). Nevertheless, protein level profiles of both lines were more related to nematode infection than to fungal infection based on the low distance between treatments. Responses to the M and FM treatments (both with gall sampling) were associated in the same cluster whereas the response to the F treatment was located in a different cluster together with the C treatment (control) (Fig. 4A). Separate analyses of the total amount of differential proteins in each chickpea line showed a similar division of treatments into two main clusters: an M+FM cluster and an F+C cluster (Fig. 4B). Differences between treatments within the same cluster were greater in 'CA 336.14.3.0' than in 'ICC 14216 K' as indicated by their linkage distances in the dendrograms (especially those involving inoculation with *Ma*) (Fig. 4B). These results suggest that 'CA 336.14.3.0' and 'ICC 14216 K' differ in their response to infection by *Ma* and *Foc*-5+*Ma* rather than to *Foc*-5 alone.

Principal component analysis (PCA) revealed that 47 out of 51 potentially responsive proteins in 'CA 336.14.3.0', and 30 out of 31 potentially responsive proteins in 'ICC 14216 K', were significantly correlated (false discovery rate, FDR, <0.05) with principal components (PCs) (Table S3). A large cluster of 33 protein spots in 'CA 336.14.3.0' and 20 in 'ICC 14216 K' was associated with PC1. Of these, 15 and 18 protein spots in 'CA 336.14.3.0' were correlated positively or negatively with PC1, respectively, compared to 11 and 9 protein spots in 'ICC 14216 K'. PC1 accounted for major differences with infection by *Ma* alone or *Ma* in combination with *Foc*-5, which were indicated by spot intensity increase (for spots correlated with PC1+) or decrease (for spots correlated with PC1–) in the M and FM treatments compared with C and F treatments. The remaining 14 protein spots in 'CA 336.14.3.0' and seven out of 10 protein spots in 'ICC 14216 K', were correlated with PC2. Twelve of the 14 'CA 336.14.3.0' spots and all seven 'ICC 14216 K' spots were correlated with PC2+. PC2 accounted for

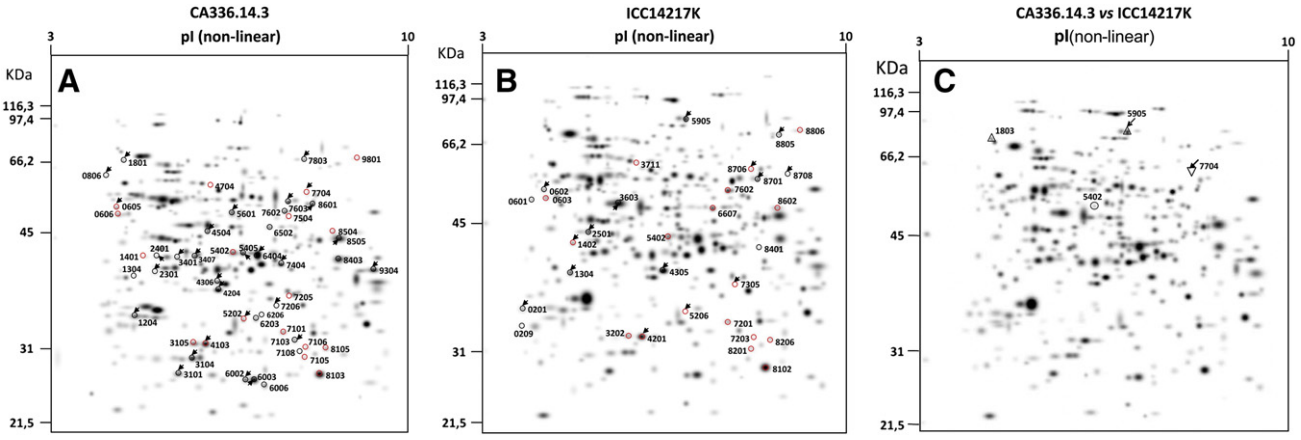


Fig. 3 – Master gels corresponding to chickpea roots extracts of: (A) Non-infected and infected 'CA 336.14.3.0' chickpeas; (B) Non-infected and infected 'ICC 14216 K' chickpeas; and (C) Control, non-infected, root tissue from 'CA336.14.3.0' and 'ICC14216K'. Molecular mass (on the left) and pI (on the top) were calculated using the PD-Quest software and standard molecular weight markers. (A) and (B): Spots marked with arrows heads showed a correct identification. Red circle: proteins affected by one/several treatments in this line. Black circle: proteins levels affected by one/several treatments in both lines. (C): Levels referred to 'CA336.14.3.0'. Triangles: higher or lower levels; circle: presence/absence of protein in some of the treatments.

major differences in plants inoculated with *Foc*-5 and particularly with *Foc*-5+*Ma*. This was indicated by spot intensity increases (for spots correlated with PC2+) or decreases (for the very small number of spots correlated with PC2–) in F and FM treatments compared with C and M treatments. Finally, the three remaining responsive spots in 'ICC 14216 K' were positively correlated with PC3+, showing increased levels of protein present in FM treatment compared with C, F and M treatments.

Protein spots that were affected in both chickpea lines generally showed similar changes in both lines. Thus, 14 out of the 18 protein spots that changed in abundance in both lines were either up or down regulated in both 'CA 336.14.3.0' and 'ICC 14216 K'. Only one commonly regulated spot (referred as CA5202 for 'CA 336.14.3.0' and IC5206 for 'ICC 14216 K' in Tables 1 and 2, respectively) showed a different pattern in the two lines, the former being negatively correlated and the latter positively correlated with PC1. Consequently, the common protein spots altered by pathogen infection would be illustrative of mechanisms underlying similar responses of resistance to *Foc*-5 in the F treatment or susceptibility to *Ma* in the M and FM treatments.

Pairwise comparisons of protein amount levels in 'CA 336.14.3.0' chickpeas resulted in 14, 23, and 32 protein spots significantly modified by F, M, and FM treatments compared with the control, respectively (Fig. 5). Similar comparisons in 'ICC 14216 K' indicated that 16, 14 and 20 protein spots were significantly modified by F, M and FM *vs*. C treatments, respectively (Figs. 5 and 6). These findings suggest that 'CA 336.14.3.0' is more responsive than 'ICC 14216 K' to infections by *Ma* as well as by *Ma*+*Foc*-5 but responds to infection by *Foc*-5 in the same way. However, comparisons using the proportion of proteins responsive to a given treatment relative to the total number of regulated proteins in each line would suggest that 'ICC 14216 K' is more responsive to infections by *Foc*-5 than 'CA 336.14.3.0' (about 53% *vs*. 30% of responsive proteins, respectively) whereas both lines have similar responsiveness to infections by *Ma* (about 47% *vs*. 49%, respectively) as well as by *Ma*+*Foc*-5 (about 63% *vs*. 68%, respectively). Also, it is worth noting that the majority of responsive proteins in both lines were up-regulated following infection either by *Foc*-5 (11 out of 14 total F-responsive proteins in 'CA 336.14.3.0' and 14 out the 16 ones in 'ICC 14216 K') or *Ma*+*Foc*-5 (28 out of 32 total FM-responsive proteins in 'CA 336.14.3.0' and 17 out the 20 ones in 'ICC 14216 K'). Conversely, the total number of M-responsive proteins was more evenly distributed between up- and down-regulations (11 and 12 proteins for 'CA 336.14.3.0', respectively; 8 and 6 proteins for 'ICC 14216 K', respectively).

Protein spots significantly modified by experimental treatments are shown in Fig. 3A–B. Most of changes in protein amount were quantitative, only 10 protein spots in 'CA 336.14.3.0' and seven protein spots in 'ICC 14216 K' were modified qualitatively by any of the treatments. Of the 10 protein spots qualitatively regulated in 'CA 336.14.3.0', six disappeared following infection by either *Foc*-5 (spots CA2401 and CA8504) or *Ma* (spots CA806, CA6006, CA6203 and CA6206), whereas four newly appeared after infections by *Ma* and *Ma*+*Foc*-5 (spots CA4704 and CA7205) or only after *Ma*+*Foc*-5 infection (spots CA1304 and CA7108). Of the seven protein spots qualitatively regulated in 'ICC 14216 K', four disappeared after infection by either *Foc*-5 (spots IC8602 and IC8708), or *Ma* and *Ma*+*Foc*-5 (spots IC201 and IC209), whereas three protein spots newly appeared after infection by *Ma* and *Ma*+*Foc*-5 (spots IC7305 and IC8706) or after infection by *Ma* (spot IC3711). As above qualitative amount of protein included four protein spots commonly regulated in both lines. Two proteins (spots CA7205/IC7305 and CA8504/IC8602) were similarly regulated in each line, while differences between lines were recorded in the two others (spots CA4704/IC3711 and CA7704/IC8706). Thus, on the one hand, CA4704 was newly detected in 'CA 336.14.3.0' after infection by *Ma* and *Ma*+*Foc*-5 whereas its homologous IC3711 was only detected in *Ma*-infected 'ICC 14216 K' plants. On the other hand, CA7704 increased his level compared to control and *Foc*-5-single infected plants after infection by *Ma* and *Ma*+*Foc*-5, whereas its homologous IC8706 in 'ICC 14216 K' was only detected after by *Ma* and *Ma*+*Foc*-5.

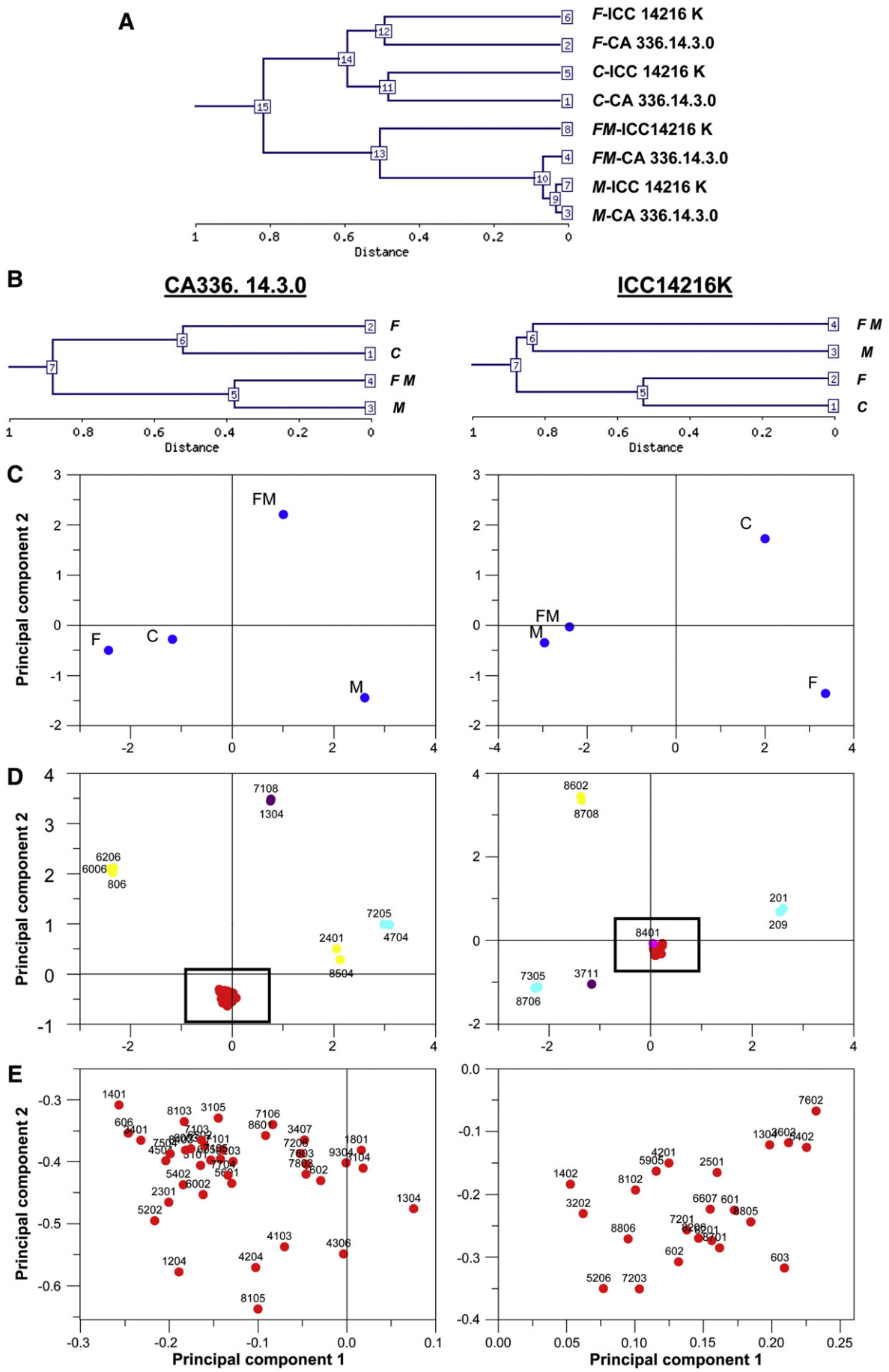
3.3. *Identified responsive proteins and major cellular functions involved in the chickpea: Foc-5:M. artiellia interaction*

Of the protein spots that were potentially differentially-expressed associated with chickpea lines and/or treatments, 64 were selected for protein identification by MALDI-TOF/TOF. The obtained PMFs and MS/MS data, and searching against MSDB using MASCOT (MatrixScience, UK), allowed identification of 36 proteins (*i.e*., a success rate of 56.3%) (Tables 1 and 2). Identified proteins included 21 and nine proteins spots specifically regulated in 'CA336.14.3.0' and 'ICC14216K', respectively, as well as six protein spots commonly regulated in both chickpea lines. Of these 36 proteins, nine were identified by matching with *C. arietinum* protein sequences, 10 by matching with protein sequences from related legume species, and 17 by sequence homology with proteins from more distantly related plant species. The relatively low success of protein identification could be a consequence of the low amount of protein used for electrophoresis separation (100 μg) because of difficulties in the gall-sampling procedures, and/or the scarcity of chickpea gene and protein sequences available in databases.

The identified proteins were classified into eight major groups according to their biological functions (Tables 1 and 2). The main functions modified in 'CA 336.14.3.0' were stress and defense responses and metabolism, each with six differentially quantified proteins, followed by nucleotide-binding proteins (four proteins), secondary metabolism and electron transport (each with three proteins), and transport, protein synthesis and protein degradation (each with one protein) (Table 1). Conversely, in 'ICC 14216 K' the main function modified was nucleotide-binding (five proteins) followed by stress and defense responses, electron transport, and metabolism (each with three proteins), and protein synthesis (one protein) (Table 2).

3.4. *Identified proteins commonly affected in chickpea lines 'CA336.14.3.0' and 'ICC14216K'*

The six proteins commonly affected in these two lines concerned stress and defense responses (three proteins), metabolism (one protein) and nucleotide binding (two proteins). Sequence similarity of the stress and defense response



proteins identified them as a resistance (R) protein of the NBS-LRR class (spot CA605/IC602), ascorbate peroxidase (APX) (spot CA4103/IC4201) and class I chitinase (spot CA5202/IC5206). All three proteins showed changed levels in both chickpea lines. The R and APX proteins showed similar patterns in both lines but the class I chitinase varied in the two lines. Spot intensity of the R protein was increased about 2- and 4-fold in 'CA 336.14.3.0' and 'ICC 14216 K', respectively, in plants infected by both *Foc*-5 and *Ma*+*Foc*-5 compared with the control (Tables 1 and 2). The R protein may be involved in specific recognition of *Foc*-5 by both chickpea lines. R proteins of the NBS-LRR class have been consistently involved in the plant–pathogen recognition mechanism underlying gene-for-gene race-specific resistance [24], although some proteomic studies revealed an apparent induction of proteins of this kind in the plant response to non-pathogenic endophytic organisms [21] and abiotic stresses [25].

Infection by *Ma*+*Foc*-5 increased the APX level by similar amounts in both chickpea lines compared with the control (Tables 1 and 2). This behavior suggests that increasing levels of APX are involved in the response to *Ma*, rather than in the differential response to *Foc*-5 that both lines showed in this treatment. However, it is also possible that this regulation had a different meaning in both chickpea lines, and thus be involved in the susceptible reaction to *Foc*-5 in 'CA 336.14.3.0' but in the resistant reaction to *Foc*-5 in 'ICC 14216 K'. APX is important for H_2O_2 detoxification in plants and hence it can be up-regulated under oxidative stress conditions. Thus, a high level of APX may be considered either the cause of a low level of H_2O_2 or, conversely, a response to an accumulation of H_2O_2. In the former case, the increase in APX should be related to a susceptible response to infection (H_2O_2 accumulation in an oxidative burst is a typical plant defense response to pathogens). Alternatively, an increase could be related to a resistant response to infection. A positive regulation of APX has been associated with a susceptible reaction in some plant–pathogen interactions [26]. In previous studies, total APX activity increased upon infection of *Foc*-5-suceptible 'JG62' chickpeas but not of *Foc*-5-resistant 'WR315'[27], which agrees with the lack of response to the F treatment in the *Foc*-5-resistant 'CA 336.14.3.0' and 'ICC 14216 K' chickpeas in the present work. Conversely, apoplastic APX activity was increased in the incompatible 'WR315'/*Foc*-5 interaction but not in the compatible 'JG62'/*Foc*-5 one [28]. Induction of APX, as that found in 'CA 336.14.3.0' and 'ICC 14216 K' after FM treatment has been also observed after infection of susceptible tomato genotypes by *Meloidogyne* spp. [29].

Class I chitinase was induced significantly in 'ICC 14216 K', but not in 'CA336.14.3.0', following infection by *Foc*-5, *Ma*, and *Ma*+*Foc*-5 compared with the control (Tables 1 and 2). In chickpea and tobacco, intracellular class I basic chitinases were shown to have higher antifungal activity than extracellular class II or class III acidic chitinases [30,31]. In chickpea, chitinase activity increased during the early stages of infection by *Foc* [32,33]. The implication of plant chitinases as defense mechanism against root-knot nematodes is not clear, although chitinases together with other extracellular hydrolases were considered important virulence factors of nematophagous fungi against plant-parasitic nematodes [34]. In *Festuca arundinaciea*, *M. marylandi* did not significantly modify the chitinase activity in the infected roots but a systemic induction occurred in other plant organs [35]. Conversely, a localized increase in chitinase activity and early induction of specific chitinase isozymes were found in soybean roots associated with resistance against *M. incognita*[36]. In the present study, the induction of chitinase was the main differential response between infected 'CA 336.14.3.0' and 'ICC 14216 K' chickpeas. This differential response could thus be interpreted as a key determinant of the maintenance of *Foc*-5-resistance in 'ICC 14216 K' compared with the overcoming of this resistance in 'CA336.14.3.0' after infection with both pathogens.

The other three protein spots commonly affected in both chickpea lines were identified as one enzyme with key anaplerotic and anabolic functions in plant primary metabolism (phosphoenolpyruvate carboxylase — PEPC) (CA7602/IC7602), and two nucleotide binding proteins, *e.g.*, probable retrotransposon protein of subclass Ty3-gypsy (CA7205/IC7305) and hypothetical protein with a B3 domain (CA7704/IC8706). Infection by *Ma* reduced PEPC by 0.3 fold in 'CA336.14.3.0' and 0.2 fold in 'ICC 14216 K', compared with the control. To the best of our knowledge, this is the first report of PEPC down-regulation being possibly involved in susceptible reaction to plant-parasitic nematodes. In legumes, such a response should be detrimental since PEPC seems to play a central role in carbon metabolism in mutualistic root nodules [37]. The spots putatively identified as Ty3-gypsy

Fig. 4 – Statistical protein amount cluster analysis of responses in chickpea lines to infection by *Fusarium oxysporum* f. sp. *ciceris* race 5 and *Meloidogyne artiellia* using the ANOVA-based array analysis tool ([22]). A–B. Dendograms showing hierarchical clustering of experimental conditions in genetic background roots with regards to un-inoculated or inoculated with pathogens (individually or simultaneously) at 35–40 DAI. A. Protein spots affected in common in both lines; B) total regulated protein spots in line CA336.14.3.0 and line ICC14216K. The amount of protein clusters are numbered from 1 to 15 in A, and from 1 to 7 in B. The statistical amount of protein cluster analysis was done using the ANOVA-based NIA-array analysis tool ([22]).
C–E. Two-dimensional bi-plots showing associations between experimental root samples and protein spots generated by principal component analysis (PCA). Line CA 336.14.3.0 (left); Line ICC 14216 K (right). Protein spots (C) were plotted in the first two components space. A short distance between root samples and protein spots in the component space is indicative of similarity in protein amount profiles. The different symbols in panel C indicate the average protein amount level in decreasing order for circle, open circle, triangle, diamond and square, respectively. Abbreviations: C = plants un-infected; M = plants inoculated with 20 eggs+J2/cm^3 of soil with *M. artiellia*; F = plants inoculated with 30,000 chlamydospores of *F. oxysporum* f. sp. *ciceris* race 5/g of soil; FM = plants inoculated with 30.000 chlamydospores of *F. oxysporum* f. sp. *ciceris* race 5/g of soil and 20 eggs +J2 of *M. artiellia*/cm^3 of soil simultaneously. Protein spots with statistically valid correlation to PCs are highlighted by their spot identification number.

Table 1 – List of differential proteins identified in roots of 'CA 336.14.3.0' chickpeas infected by *Fusarium oxysporum* f. sp. *ciceris* race 5 and/or *Meloidogyne artiellia*.

Spot no. [a]	Protein name	Plant species Accession no. [b]	MASCOT scores [c]				Mr (kDa)/pI [e]	Fold change [f]	
			Protein score/ peptide count	Protein score CI%	Total ion score [d]	Total ion score CI%			
Stress and defense response [g]									
CA605/IC602 [h]	Putative citrus disease resistance protein Pt3 (Fragment)	*Citrus grandis x Poncirus trifoliate* (Q8H203) gi|22947659	70/8	99.77	–	–	52.8/5.5	F/C=2.0; FM/C=2.3	M/F=0.4; FM/M=2.8
CA4103/IC4201 [h]	Ascorbate peroxidase (Fragment)	*Cicer arietinum* (Q9SXT2) gi|4586574	90/3	100	69	100	32.3/6.8	FM/C=4.2	FM/F=3.5; FM/M=2.1
CA4504	Peroxidase (EC1.11.1.7) pxdC (precursor)	*Medicago sativa* (T09665) gi|537317	101/4	100	85	100	47.0/6.8	M/C=5.5	M/F=3.7; FM/M=0.4
CA5202/IC5206 [h]	Class I chitinase (precursor) (EC 3.2.1.14)	*Cicer arietinum* (Q9ZP10) gi|3892724	86/11	100	–	–	35.1/7.1	–	M/F=0.2; FM/M=3.5
CA8601	Catalase (EC 1.11.1.6)	*Mesmembryanthemum crystallinum* (T12300) gi|3202032	196/15	100	88	100	55.4/8.0	M/C=0.5	M/F=0.3; FM/M=3.2
CA9304	NBS-LRR disease resistance protein-like (protein-like)	*Oryza sativa* (Q9LJ10) gi|75335055	84/18	100	–	–	41.3/9.3	FM/C=3.8	FM/F=2.2; FM/M=2.9
Signal transduction [g]									
CA1204	Plasma membrane intrinsic polypepetide	*Cicer arietinum* (Q9SMK5) gi|6469121	199/18	100	–	–	34.9/5.7	MF/C=0.3	MF/F=0.4
CA2301	Annexin (fragment)	*Medicago sativa* (T09552) gi|512400	69/9	99.71	–	–	40.4/6.0	F/C=0.3; FM/C=2.2	M/F=4.4; FM/F=7.7
Electron transport [g]									
CA1801	Cytochrome P450-like protein (Fragment)	*Arabidopsis thaliana* (Q681L2) gi|51969352	72/10	99.85	–	–	68.3/5.6	M/C=0.2	M/F=0.1; FM/M=8.0
CA6002	Probable quinone oxidoreductase (EC 1.6.5.5).-	*Cicer arietinum* (Q8L5Q7) gi|21068664	78/9	99.96	–	–	26.9/7.1	FM/C=1.9	FM/M=1.8
CA6003	Probable quinone oxidoreductase (EC 1.6.5.5).-	*Cicer arietinum* (Q8L5Q7) gi|21068664	167/12	100	46	99.94	26.9/7.2	–	M/F=0.5; FM/M=1.7
Metabolism [g]									
CA2401	Glutamine synthetase	*Medicago trunculata* (O04998) gi|1835154	78/6	99.97	48	99.96	42.7/6.0	F/C=0; M/C=1.7; FM/C=3.6	M/F=∞; FM/F=∞; FM/M=2.1
CA3104	Triosephosphate isomerase	*Glycine max* (Q38IW8) gi|77540216	151/9	100	82	100	30.7/6.6	F/C=1.9; FM/C=1.9	M/F=0.4; FM/M=2.6
CA3401	Glutamine synthetase root isozyme A	*Pisum sativum* (P07694) gi|121333	148/9	100	90	100	42.6/6.3	FM/C=2.6	M/F=0.4; FM/M=4.6
CA5601	Phosphogluconate dehydrogenase (decarboxilating) (EC 1.1.1.44)	*Glycine max* (O22111) gi|2529229	236/15	100	133	100	52.0/6.9	M/C=0.4	M/F=0.4; FM/M=2.4

CA7602/IC7602[h]	Phosphoenolpyruvate carboxylase	*Zea mays* (X61489) gi|162458345	72/16	99.84	–	–	52.5/7.5	M/C=0.3	M/F=0.3; FM/M=2.7
CA7603	Serine hydroxymethyltransferase	*Populus tremoloides* (EF150637) gi|134142065	204/14	100	106	100	55.1/7.5	M/C=2.1; FM/C=2.7	M/F=2.2; FM/F=2.8
Secondary metabolism[g]									
CA3101	Chalcone isomerase (Fragment)	*Cicer arietinum* (Q9SXS9) gi|4586568	127/11	100	–	–	27.5/6.3	M/C=0.4	M/F=0.3
CA4204	Isoflavone reductase-like NAD(P)H-dependent oxidoreductase	*Medicago sativa* (Q9SDZ7) gi|6525021	152/4	100	135	100	38.4/6.9	–	M/F=2.1
CA4306	2′-hydroxyisoflavone reductase (EC 1.3.1.45)	*Cicer arietinum* (S17830) gi|1708425	152/15	100	–	–	39.4/6.8	FM/C=0.5	FM/M=0.6
Transport[g]									
CA3407	Myosin heavy chain-like protein	*Arabidopsis thaliana* (Q9FJ35) gi|10177363	82/19	99.99	–	–	42.8/6.6	M/C=0.4	M/F=0.2; FM/M=4.1
CA8505	Myosin heavy chain-like	*Arabidopsis thaliana* (Q9FJ36) gi|15238179	77/13	99.96	–	–	46.6/8.4	–	FM/M=0.5
Protein synthesis and degradation[g]									
CA7103	Proteasome subunit alpha type-7	*Cicer arietinum* (Q9SXU1) gi|12229936	283/19	100	69	100	32.9/7.6	F/C=1.9; FM/C=2.7	M/F=0.5; FM/M=2.8
Nucleotide-binding[g]									
CA7205/IC7305[e]	Probable retrotransposon, subclaseTy3-gypsy	*Oryza sativa* (Q53MU3) gi|62734737	87/21	100	–	–	37.7/7.5	M/C=∞; FM/C=∞	M/F=∞; FM/F=∞; FM/M=2.6
CA7704/IC8706[h]	B3 domain-containing protein	*Arabidopsis thaliana* (Q5PNU4) gi|75107723	76/10	99.94	–	–	59.2/8.4	M/C=2.4; FM/C=4.3	FM/F=2.4; FM/M=1.8
CA7803	Probable gag-pol polyprotein	*Solanum demissum* (Q5NRP7) gi|56744299	71/22	99.83	–	–	71.7/7.8	–	FM/M=2.1
CA8403	RNA recognition motif (RRM)-containing protein	*Arabidopsis thaliana* (Q3EBP3) gi|30685698	89/18	100	–	–	43.0/8.4	FM/C=0.5	FM/F=0.5; FM/M=0.3

[a] Spot numbers as in Fig. 3.
[b] Accession number of the protein according to NCBInr database.
[c] MOWSE Score probability (Protein score) for the entire protein and for ions, complemented by the percentage of the confidence interval (CI).
[d] Information about peptide fragmentation when available.
[e] Experimental mass values were calculated with PD-Quest™ software and standard molecular mass markers, while pI was calculated manually by recognizing characteristic spots in other gels for reference.
[f] Values of fold change between the normalized volume of inoculated tissue, *F. oxysporum* f. sp. *ciceris* race 5 (F), *M. artiellia* (M), *F. oxysporum* f. sp. *ciceris* race 5 — *M. artiellia* (FM), and control (C) tissue, e. g. ratio F/C, *F.oxysporum* f. sp. *ciceris* race 5 *versus* control tissue. "∞" indicates that the spot is not detected in the assay denominator. "–" indicates no differences in the comparisons between these treatments.
[g] Proteins were classified based on their major functions appeared in Swiss-Prot and TrEMBL database as well as literature search in the PubMed. Some proteins might have multiple functions but were put into only one functional category in this Table.
[h] Protein spots affected by the treatments in both lines, the first number is the gel identification number (CA) in line 'CA336.14.3.0', followed by IC in 'ICC14216K'.

Table 2 – List of differential proteins identified in roots of 'ICC14216K' chickpeas infected by *Fusarium oxysporum* f. sp. *ciceris* race 5 and/or *Meloidogyne artiellia*.

Spot no. [a]	Protein name	Plant species Accession no. [b]	MASCOT scores [c]				Mr (kDa)/pI [e]	Fold change [f]	
			Protein score/ peptide count	Protein score CI%	Total ion score [d]	Total ion score CI%			
Stress and defense response [g]									
IC6602/ CA605 [h]	Putative citrus disease resistance protein Pt3 (Fragment)	*Citrus grandis x Poncirus trifoliate* (Q8H203) gi|22947659	70/8	99.77	–	–	52.8/5.5	F/C=3.7; FM/ C=4.1	M/F=0.5; FM/ M=2.3
IC4201/ CA4103 [h]	Ascorbate peroxidase (Fragment)	*Cicer arietinum* (Q9SXT2) gi| 4586574	90/3	100	69	100	32.3/6.8	FM/C=5.0	M/F=0.5; FM/ F=3.7; FM/M=6.9
IC5206/ CA5202 [h]	Class I chitinase (precursor). — (EC 3.2.1.14)	*Cicer arietinum* (Q9ZP10) gi| 3892724	86/11	100	–	–	35.1/7.1	F/C=4.6; M/C=4.7; FM/C=9.8	–
Electron transport [g]									
IC2501	Probable protein disulfide-isomerase A6. Precursor	*Medicago sativa* (P38661) gi|729442	134/16	100	–	–	43.9/6.0	–	M/F=0.5; FM/ M=2.4
IC8701	NADH dehydrogenase	*Solanum tuberosum* (S52261) gi| 639834	151/19	100	–	–	55.4/8.0	F/C=3.6; FM/ C=2.8	M/F=0.4; FM/ M=2.1
IC8805	Os08g0226300 protein	*Oryza sativa* (Q0J767) gi|115475375	93/22	100	–	–	71.1/8.4	F/C=2.9	M/F=0.3; FM/ M=2.7
Metabolism [g]									
IC3603	S-adenosylmethionine synthetase	*Pisum sativum* (Q76KV5) gi| 223635320	230/23	100	–	–	49.4/6.5	M/C=0.3	M/F=0.2; FM/ M=3.2
IC5905	Methionine synthase	*Glycine max* (Q71EW8) gi|33325957	90/16	100	–	–	81.7/7.1	FM/C=3.9	FM/F=2.8; FM/ M=3.8
IC7602/ CA7602 [h]	Phosphoenolpyruvate carboxylase	*Zea mays* (X61489) gi|162458345	72/16	99.84	–	–	52.5/7.5	M/C=0.2	M/F=0.2; FM/ M=5.2
Protein synthesis and degradation [g]									
IC8708	Putative extracellular dermal glycoprotein	*Cicer arietinum* (Q9FSZ9) gi|	94/12	100	–	–	57.3/8.62	F/C=0; FM/C=4.3	M/F=∞; FM/F=∞

	(Fragment)		10334495						
Nucleotide-binding[g]									
IC201	AP2/ERF and B3 domain-containing transcription factor At1g51120	*Arabidopsis thaliana* (Q9C688) gi|15223743	72/10	99.86	–	–	35/4.66	F/C=3.0 M/C=0; FM/C=0	M/F=0; FM/F=0;
IC1304	Putative sister-chromatide cohesion protein	*Arabidopsis thaliana* (Q8LFH0) gi|21537070	96/19	100	–	–	39/5.82	M/C=0.4	M/F=0.3; FM/M=2.8
IC7305/CA7205[h]	Probable retrotransposon, subclaseTy3-gypsy	*Oryza sativa* (Q53MU3) gi|62734737	87/21	100	–	–	37.7/7.5	M/C=∞; FM/C=∞	M/F=∞; FM/F=∞; FM/M=2.5
IC8401	SMC1 Protein	*Oryza sativa* (Q8GU56) gi|27227801	93/21	100	–	–	41.8/8.0	–	M/F=3.6; FM/F=4.2
IC8706/CA7704[h]	B3 domain-containing protein	*Arabidopsis thaliana* (Q5PNU4) gi|75107723	76/10	99.94	–	–	59.2/8.4	M/C=∞; FM/C=∞	M/F=∞; FM/F=∞

[a] Spot numbers as in Fig. 3.
[b] Accession number of the protein according to NCBInr database.
[c] MOWSE Score probability (Protein score) for the entire protein and for ions, complemented by the percentage of the confidence interval (CI).
[d] Information about peptide fragmentation when available.
[e] Experimental mass values were calculated with PD-Quest™ software and standard molecular mass markers, while pI was calculated manually by recognizing characteristic spots in other gels for reference.
[f] Values of fold change between the normalized volume of inoculated tissue, *F. oxysporum* f. sp. *ciceris* race 5 (F), *M. artiellia* (M), *F. oxysporum* f. sp. *ciceris* race 5 — *M. artiellia* (FM), and control (C) tissue, e. g. ratio F/C, *F. oxysporum* f. sp. *ciceris* race 5 *versus* control tissue. "∞" indicates that the spot is not detected in the assay denominator. "–" indicates no differences in the comparisons between these treatments.
[g] Proteins were classified based on their major functions appeared in Swiss-Prot and TrEMBL database as well as literature search in the PubMed. Some proteins might have multiple functions but were put into only one functional category in this Table.
[h] Protein spots affected by the treatments in both lines.

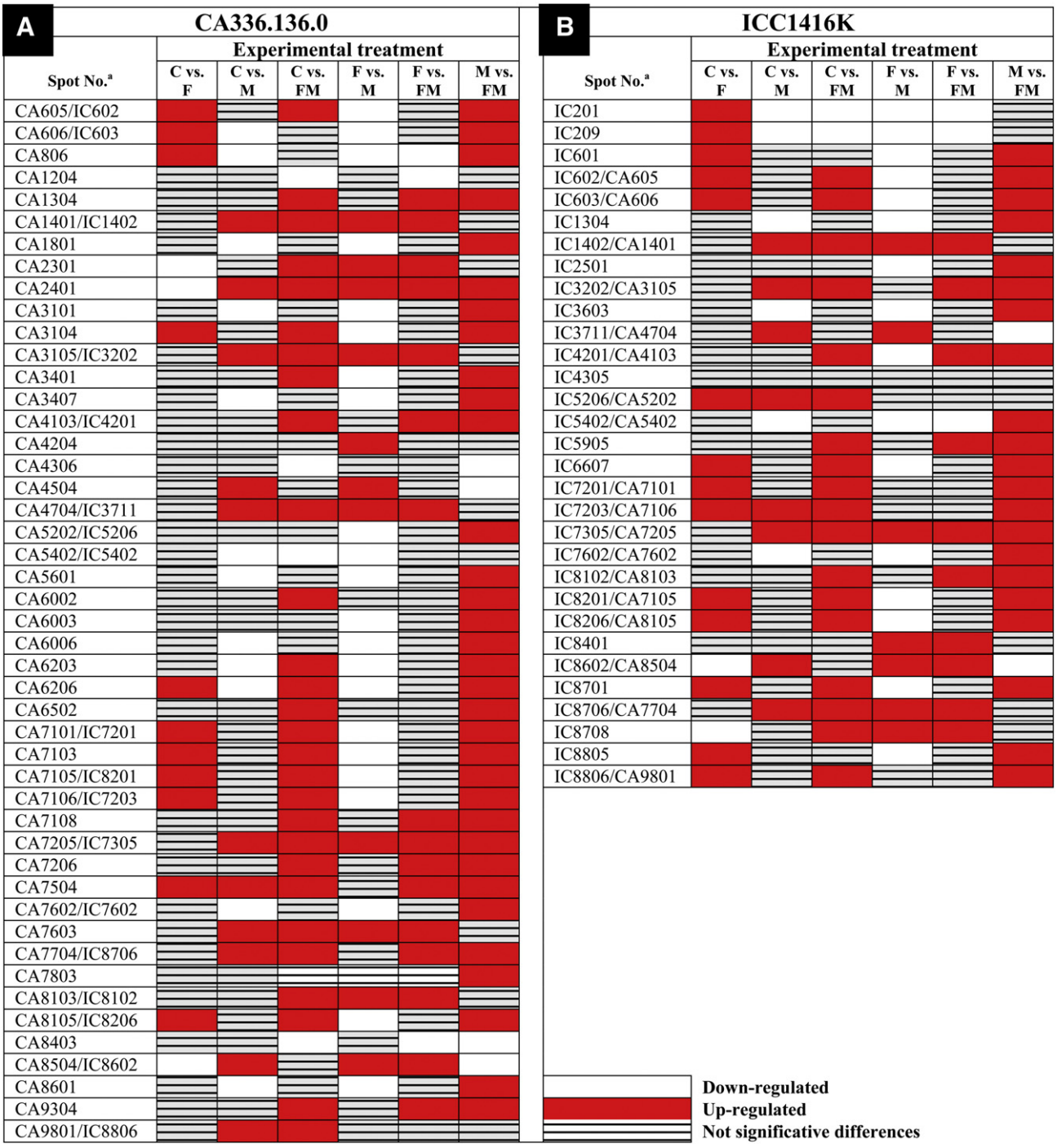


Fig. 5 – Pairwise comparisons of protein spots values in 'CA336.1.4.3.0' (A) and 'ICC14216K' (B). The three first columns are the pairwise comparisons with the control in order to detect the pathogen effect on plants, while the others comparisons are used to detect additive effect between pathogen inoculations. Abbreviations: C = Control; F = *Fusarium oxysporum* f. sp. *ciceris* race 5; M = *Meloidogyne artiellia*; FM = both pathogens. [a]Protein spots are numbered as in Fig. 3.

retrotransposon and B3-domain proteins became up-regulated in response to infection of 'CA336.14.3.0' and 'ICC 14216 K' chickpeas by *Ma* (both in M and FM treatments), suggesting a role for these changes in the susceptible reaction to the nematode. The Ty3-gypsy spot represented a new appearance in both chickpea lines, whereas the differences in the B3-domain protein were related to differential amount between treatmentsin 'CA336.14.3.0', but new appearance in M+FM treatments in 'ICC 14216 K'. Retrotransposons, a class of transposable elements that encode reverse transcriptase and propagate like retroviruses via a RNA intermediate, are activated by a variety of biotic and abiotic stresses [38]. B3 DNA binding domains are shared by numerous plant-specific transcription factors, including those involved in transcription regulated by auxins and other phytohormones [39]. Assuming such a role for CA7704/IC8706, the fact that this

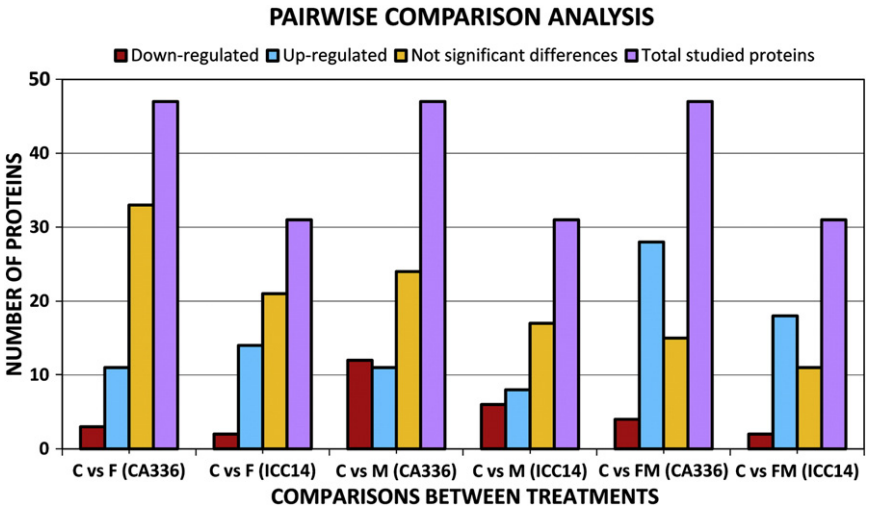


Fig. 6 – Number of proteins from pairwise comparisons of protein spots values in 'CA336.14.3.0' (CA336) and 'ICC14216 K' (ICC14).

protein is up-regulated following *Ma* infection in the two chickpea lines suggests a role in the transcriptional activation of genes involved in nematode gall formation/maintenance.

3.5. *Identified proteins specifically affected in their levels by pathogen infection in only one of the chickpea lines*

Most of identified responsive proteins (21 in 'CA336.14.3.0' and nine in 'ICC14216K') were regulated specifically in one of these chickpea lines. It is unlikely that these line-specific changes would describe general aspects of infection by either *Foc*-5 or *Ma*, since those two chickpea lines share a similar root proteome and showed same resistant or susceptible reaction to both pathogens, respectively. Nevertheless, they might relate to specific aspects of chickpea/*Foc*-5 or *Ma* interactions or the differential response of 'CA336.14.3.0' and 'ICC14216K' to co-infection by the two pathogens. These proteins will be briefly described grouping them according to their biological functions.

3.5.1. *Stress and defense response*

This group of proteins included three 'CA336.14.3.0'-specific amount changes that were identified as: (i) a class III peroxidase (spot CA4504), (ii) a catalase (spot CA8601), and (iii) a R protein of the NBS-LRR class (spot CA9304) (Table 1). The class III peroxidase increased 5.5 fold after *Ma* infection compared with the control; whereas the catalase decreased 0.5 fold in intensity after the same infection. Conversely, the NBS-LRR-class protein increased about 4 fold after *Foc*-5 + *Ma* infection compared with the control, but did not change after infection by either pathogen alone. The class III peroxidase and catalase level changes reinforce the formerly suggested redox perturbation associated with chickpea response to *Ma* infection as described above for common regulation of an APX.

3.5.2. *Signal transduction*

This group of proteins included two 'CA336.14.3.0' specific level changes that were identified as: (i) a plasma membrane (PM) intrinsic polypeptide (spot CA1204) and (ii) an annexin (spot CA2301). The PM polypeptide levels were lowered in chickpeas infected by *Foc*-5 + *Ma* compared with the control but were not influenced by individual infection by each of the pathogens. Conversely, compared with the control the annexin protein levels were reduced following *Foc*-5 infection but increased in plants infected with *Foc*-5 + *Ma* (Table 1). Plant annexins are Ca^{2+}- and phospholipid-binding, predominantly cytosolic proteins that are involved, among other functions, in signaling pathways mediated by cytosolic calcium and reactive oxygen species [40]. Similar binding properties and function in signaling pathways have been assigned to some plant root PM intrinsic polypeptides [41]. The differential regulation of both proteins following infections by *Foc*-5 + *Ma* or *Foc*-5 alone would support the hypothesis that they are involved in the susceptible reaction of the *Foc*-5-resistant 'CA336.14.3.0' induced by co-infection with *Ma*.

3.5.3. *Electron transport*

Several proteins specifically regulated in either of the chickpea lines were identified as oxido-reductases and are thus included in this group despite their functional diversity. The 'CA 336.14.3.0'-specific level changes included three spots that were identified as: (i) a cytochrome P450 type protein (spot CA1801), and (ii) a single quinone oxidoreductase (QOR) (spots CA6002 and CA6003) (Table 1). The 'ICC14216K'-specific level changes also included three spots (Table 2), which were identified as: (i) a putative protein disulfide isomerase (PDI; spot IC2501), (ii) a NADH dehydrogenase (NDH; spot IC8701), and (iii) a protein functionally related to CA1801 (spot IC8805) from which it differed in electrophoretic properties. All the above enzymes may be involved in the reaction to stressful conditions, either catalyzing critical hydroxylating steps in the biosynthesis of antimicrobial and antioxidant secondary metabolites such as isoflavonoid phytoalexins in chickpeas (as is the case for cytochromes P450; [42]), acting as antioxidant enzymes (as for QOR and NDH; [43]), or assisting in the folding of defense proteins in (the case of PDI; [44]). However, in the present study the regulation of the above proteins only involved small changes in quantity and/or it was only produced in a minority of the treatments, suggesting that such level changes are

probably an effect rather than the cause of the susceptible or resistant reactions in which they are involved.

3.5.4. *Metabolism*

This group of proteins included five specific spots in 'CA 336.14.3.0' (Table 1) and two in 'ICC14216K' (Table 2). In 'CA 336.14.3.0', the identified proteins included two isoforms of glutamine synthetase (GS; spots CA2401 and CA3401), a triosephosphate isomerase (TPI; spot CA3104), a phosphogluconate dehydrogenase (PGDH; spot CA5601), and a serine hydroxymethyltransferase (SHMT; spot CA7603). Thus, the affected proteins concerned key steps in nitrogen and amino acid metabolism (GS and SHMT), carbohydrate metabolism (TPI and PGDH), and one-carbon metabolism (SHMT). In 'ICC14216K', the proteins which levels were modified were identified as an S-adenosylmethionine synthetase (SAM synthetase; spot IC3603) and a methionine synthase (Met synthase; spot IC5905). These enzymes are involved in amino acid metabolism (Met synthase) and one-carbon metabolism (SAM synthetase and Met synthase). One-carbon metabolism is of importance in plant defense since transmethylation reactions are involved in the biosynthesis of the plant hormone ethylene, lignin precursors, or phytoalexins. In our study, infection of 'CA 336.14.3.0' chickpeas by *Ma* and *Foc*-5+*Ma* increased SHMT 2 to 3 fold compared with the control (Table 1). Conversely, infection of 'ICC14216K' by *Ma* reduced SAM synthetase 0.3 fold and that by *Foc*-5+*Ma* increased Met synthase 4 fold, compared with the control (Table 2). These results suggest a significant role for the transmethylation reaction in the chickpea:*Ma*:*Foc*-5 interaction.

The two spots identified as GS in 'CA 336.14.3.0' were up-regulated in plants infected by *Foc*-5+*Ma* while they were either not detected (CA2401) or not significantly affected (CA3401) in plants infected by *Foc*-5 (Table 1). GS plays a central role in plant nitrogen assimilation and recycling, being a key factor regulating plant responses to nitrogen availability and stress conditions [45]. The glycolytic TPI was similarly increased approximately 2 fold in 'CA 336.14.3.0' infected by *Foc*-5 and *Foc*-5+*Ma* compared with the control (Table 1), whereas PGDH decreased 0.4 fold that of the control in plants infected by *Ma* (Table 1). This latter down-regulation might contribute to susceptibility of 'CA 336.14.3.0' chickpea to *Ma*. PGDH, a rate-limiting enzyme in the pentose phosphate pathway, provides NADPH required for NADPH oxidase activity at the plasma membrane for producing ROS during plant defense responses against pathogens [46]. Nevertheless, PGDH activity in root was found to increase both in cotton resistant and susceptible after infection by *M. incognita*[47].

3.5.5. *Secondary metabolism*

Three protein spots specifically regulated in 'CA 336.14.3.0' chickpeas were identified as enzymes of the flavonoid/isoflavonoid pathway: chalcone isomerase (CHI; spot CA3101), isoflavone reductase (IFR; spot CA4204) and 2′-hydroxyisoflavone reductase (2′HFR; CA4306) (Table 1). Chalcone isomerise, along with chalcone synthase, is the entry point of cinnamic acids from the phenylpropanoid pathway into the flavonoid/isoflavonoid biosynthesis pathway. IFR and 2′HFR are isoflavone reductases of similar function, or even isoforms of the same reductase that participate in the reduction of 2′-hydroxyisoflavones to isoflavones during the biosynthesis of pterocarpan phytoalexins. Maackiain and medicarpin, which belong to the pterocarpan phytoalexins, are the two main chickpea phytoalexins [48]. Production of these phytoalexins is one of the most common defense responses of chickpea to pathogens or elicitors [49] and they are produced during infection of chickpea by *Foc* in amounts that correlated with resistance [32,50]. However, it is unclear whether CHI, IFR, and 2′HFR played a defensive role in the chickpea–pathogen interaction in this study. First, their levels were lowered rather than increased, and secondly their pattern of production was not consistent with the nature of the interaction, *i.e.*, CHI and 2′HFR were decreased only following infection by *Ma* and *Foc*-5+*Ma*, respectively, compared with the control, whereas IFR levels increased after infection by *Ma* but not *Foc*-5 (Table 1). The level of phytoalexins was reported to decrease after infections by plant-parasitic nematodes, a response which has been related to susceptibility to the nematode as well as overcoming of resistance to other soil borne pathogens [51,52]. Nevertheless, infection of alfalfa roots by *M. incognita* was associated with an early transcriptional induction of an IFR both in resistant and susceptible cultivars [53].

3.5.6. *Protein synthesis and degradation*

Infection of 'CA 336.14.3.0' by *Foc*-5 and *Foc*-5+*Ma* increased the level of subunit alpha 7 of the chickpea 26S proteasome 2 to 3 fold (Table 1, spot CA7103). In 'ICC14216K', infections by *Foc*-5+*Ma* increased a putative extracellular dermal glycoprotein with inferred aspartic endoprotease activity approximately 4 fold (Table 2, spot IC8708) while this increase was not detected following infection by *Foc*-5 alone. Proteasomes are multicatalytic regulatory proteases that operate on mis-folded, damaged or oxidized proteins. Proteasome activity can control the plant response to stressful conditions including oxidative or biotic stress and may also control the functioning of key signal transduction cascades [54]. In our study, the increase in proteasome activity could be interpreted as a chickpea response to stress caused by *Foc*-5 infection (*i.e.*, oxidative stress and other types of stresses) that had no effect on the outcome of co--infection with *Ma* as indicated by a similar response in F and FM treatments.

3.5.7. *Nucleotide-binding*

Two specific proteins in 'CA 336.14.3.0' (spots CA7803 and CA8403; Table 1) and three specific proteins in 'ICC14216K' (spots IC201, IC1304 and IC8401; Table 2) were identified as nucleotide-binding proteins. Spot CA7803 was identified as a putative gag-pol polyprotein operating in replication of retroelements. However, its regulation was restricted to a 2 fold increase with infection by *Foc*-5+*Ma* relative to that by *Ma* alone (Table 1). More interestingly, spot CA8403 decreased after infection with *Foc*-5+*Ma* compared with infection by *Foc*-5 and the control (Table 1). This result suggests its involvement in the specific overcoming of *Foc*-5 resistance in 'CA 336.14.3.0' after infection by *Ma*. This spot was identified as a protein containing a RNA recognition motif (RRM), a class of proteins that seems to function in the post-transcriptional control of gene expression hence having crucial roles in plant stress response as well as in development and genome organization [55].

Spot IC201 in 'ICC14216K' was increased 3 fold following infection by *Foc*-5 but was not detected in plants infected by either

Ma or *Foc*-5+*Ma* (Table 2). This protein was identified as a transcription factor containing AP2/ERF and B3 domains that are unique to higher plants. While the AP2/ERF domain is apparently implicated in plant responses to biotic and abiotic stresses, the B3 domain is involved in plant responses to auxin and other plant hormones [39]. Therefore, it is possible to speculate that different domains of the protein are responsible for its roles in the resistant or susceptible reaction of 'ICC14216K' chickpeas to *Foc*-5 and *Ma*, respectively, after the increase of level or the absence of the protein induced by these pathogens. The remaining spots of nucleotide-binding proteins specifically regulated in 'ICC14216K' were identified as two cohesins functionally related to the cohesion of sister chromatides during DNA replication, namely a putative sister-chromatide cohesion protein (SCC protein; spot IC1304, Table 2) and a structural maintenance of chromosomes protein (SMC1 protein; spot IC8401, Table 2). In spite of their close functional relationship, each of these proteins showed a different pattern in their levels. Thus, infection by *Ma* decreased the SCC protein to 0.4 fold of that of the level in the control while the level of the SMC1 protein increased about 4 fold following infection by *Ma* as well as *Foc*-5+*Ma*, compared with that in infection by *Foc*-5 alone (Table 2).

3.5.8. *Transport*

Two spots specifically regulated in 'CA 336.14.3.0' were identified as a myosin heavy chain-like protein (spots CA3407 and CA8505, Table 1). Infection by *Ma* reduced the intensity of the first one to 0.4 fold of that in the control, whereas the intensity of the second one was significantly reduced 0.5 fold only after infections by *Foc*-5+*Ma* compared with the intensity in plants infected by *Ma* alone. Myosin is a molecular motor responsible for actin-based motility processes in plant cells, some of which, like cytoplasmic aggregation, may relate to defense against stresses [56]. In this regard it is of interest that actin gene expression is induced in chickpea roots in response to infection by *Foc*-5 [28]. Therefore, myosin down-regulation upon infection by *Ma* might be envisaged as a shortage in the plant defense potential and therefore as a factor contributing to susceptibility to the nematode.

3.6. *Differences in the proteomes of the chickpea lines 'CA336.14.3.0' and 'ICC14216K'*

Two of the proteins described above as being responsive to infection, methionine synthase (spot IC5905, Table 2) and B3 domain-containing protein (spot CA7704/IC8706, Tables 1 and 2) were among four protein spots that were found to be present at different levels in non-inoculated plants of the two chickpea lines. Intensity of the former protein was lower in 'CA 336.14.3.0' than in 'ICC14216K' (0.4 ratio) whereas the latter was present only in 'CA 336.14.3.0'.

4. Conclusions

To the best of our knowledge, this is the first study at the root proteome level of chickpea response to a biotic stress imposed by single and joint infections by two major soil-borne pathogens, namely the fungus *F. oxysporum* f.sp. *ciceris* race 5 (*Foc*-5) and the root-knot nematode *M. artiellia* (*Ma*). The results demonstrate the utility of our proteomic approach to unravel interesting aspects of plant–pathogen interactions, although the use of root tissues and gall-enriched root samples of a non-model, un-sequenced plant species challenged by two pathogens was not an easy experimental system. 'CA 336.14.3.0' and 'ICC 14216 K' chickpeas used in this present study yielded quite similar root proteomes. Therefore, the differential response to *Foc*-5 after co-infection by *Ma* found in the study does not appear to concern differences in constitutive protein levels. On the contrary, the root proteomes of the two chickpea lines displayed clear differences after infection by those pathogens. 'CA 336.14.3.0' chickpeas, whose resistance to *Foc*-5 is overcome by co-infection with *Ma*, displayed a higher number of responsive proteins following infections by the pathogens compared with that of 'ICC 14216 K' in which resistance is not influenced by *Ma*. That difference was due to a higher number of responsive proteins to infection by *Ma*, either alone or jointly with infection by *Foc*-5. The number of proteins responsive to infection by *Foc*-5 alone was similar in the two chickpea lines. This higher responsiveness of 'CA 336.14.3.0' chickpeas compared with 'ICC 14216 K' may relate to a stronger metabolic re-programming in the former line during infection by the nematode, which would govern the differential responses of both lines to co-infection with the two pathogens without affecting their similar susceptible response to *Ma*.

The root proteome of the two chickpea lines comprised both common and specific responses to the inoculation of both pathogens. The common responses, which came to a total of 18 protein spots, included about 40% of the total responsive proteins in 'CA 336.14.3.0 K' but 60% of those in 'ICC 14216'. Most proteins that were affected in common in both lines displayed similar patterns of proteins levels. This suggests that the proteomic approach used is consistent and reliable and also indicates that the response of 'CA 336.14.3.0' and 'ICC 14216 K' chickpeas to *Foc*-5 and *Ma* has a common basis and that proteins affected may play a key role in the plant defense response. The small number of proteins affected in common in both lines but that showed different levels of protein in each probably play important roles in the differential response that 'CA 336.14.3.0' and 'ICC 14216 K' chickpeas displayed following co-infections by the two pathogens. This is the case of a class I chitinase in the differential response of the two lines to *Foc*-5 in plants co-infected with both pathogens.

In summary, we conclude that: 1) the similar responses of 'CA 336.14.3.0 K' and 'ICC 14216' chickpeas to infection by either *Foc*-5 or *Ma* must rely both on defense reactions underlying the aforementioned common defensive basis as well as interaction mechanisms that differ between both lines; and 2) the higher number of specific protein spots levels changed in 'CA 336.14.3.0' compared with those in 'ICC 14216 K' may reflect the susceptible reaction to *Foc*-5 that only occurs in the former after co-infection by *Foc*-5 and *Ma*.

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Appendix A. Supplementary data

Supplementary materials related to this article can be found online at doi:10.1016/j.jprot.2011.05.026.

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