Uluslararası Tarım Araştırmalarında Yenilikçi Yaklaşımlar Dergisi
International Journal of Innovative Approaches in Agricultural Research 2019, Vol. 3 (1), 112-122
https://doi.org/10.29329/ijiaar.2019.188.11

Original article

Chickpea Callus Histology Inoculated with *Ascochyta Rabiei* Blight Causal Agent Spores [1]

Amina Kadiri [a, *], Zineb Faiza Boukhatem [a], Yamina Halfaoui [a] & Zohra Ighilhariz [a]

[a] Université Oran1 Ahmed Ben Bella, Nature and Life Sciences Faculty Oran, Algeria

Abstract

The objective of this research was to study the interaction between *Ascochyta rabiei* as a pathogen and its host, established through *in vitro* tissue culture using as a pathosystem chickpea *Cicer arietinum* callus inoculated with *Ascochyta rabiei* spores. A resistant chickpea genotype INRA 199 and a local cultivar "Zouaoui" were used.

Murashige and Skoog, (1962) medium supplemented with 0.5 mg/l of Naphthalene Acetic Acid (NAA) and 1 mg/l of Benzyl Amino Purine (BAP) were used for the production of calluses used as a host. The histological study of calli inoculated with *Ascocchyta rabiei* spore suspension compared to the control showed two different reactions. In INRA199, the pathogen proliferation was slow and limited by the formation of an area where the cells accumulated phenolic compounds whereas in the cultivar Zouaoui the pathogen rapidly colonized the calli intercellular space and the number of formed pycnidia was high.

Keywords: Cicer arietinum, Pathosystem, In vitro culture, Chickpea blight.

Received: 25 July 2018 * **Accepted:** 19 January 2019 * **DOI:** https://doi.org/10.29329/ijiaar.2019.188.11

* **Corresponding author:**

Amina Kadiri, Université Oran1 Ahmed Ben Bella, Nature and Life Sciences Faculty Oran, Algeria
Email: amkad71@gmail.com

[1] A part of this study was presented at the International Agricultural, Biological and Life Science Conference, Edirne, Turkey, September 2-5, 2018.

INTRODUCTION

Chickpea *Cicer arietinum* is an important grain legume grown in 50 countries (Gupta et al., 2014) on an area of more than 13 million ha with an annual production more than 13 million tons (FAO, 2015). This plant is sensitive to several abiotic and biotic factors including ascochyta blight, a cryptogamic disease caused by *Ascochyta rabiei* (Shah et al., 2015). This disease was reported in 35 countries around the world, affecting seeds quality and quantity (Gan et al., 2006) and under humid wind and rain conditions, losses can be total. In Algeria, anthracnosis is reported in all areas where chickpea is grown. The undertaken investigations reveal epidemic disease presence particularly dangerous for local cultivars. The control is mainly based on conventional methods but these approaches remain insufficient and need to be assisted by biotechnological tools to facilitate resistant plants selection. Thus, tissue culture and callogenesis associated with phytopathological techniques facilitate the *in vitro* resistant plants screening. For *Cicer arietinum*, these tools were used for tolerant genotypes calli selection to NaCl, atrazine herbicide (Mc Phee et al., 2010) and *Ascochyta rabiei* (Kumar et al., 2010). It was also used to study and understand defence mechanisms against fusariosis (Singh et al., 2007) and anthracnosis (Mc Phee et al., 2010). This latest interaction is studied in this work histologically to evaluate the host response to the parasite.

Material and Methods

Calli obtaining

INRA 199 genotype (resistant to *Ascochyta rabiei*) and a local chickpea cultivar "Zouaoui" seeds were used to produce the stem segments *in vitro* for tissue culture (Kadiri et al., 2013). They were disinfected and cultured on MS (Murashige and Skoog, 1962) medium supplemented with NAA (0.5 mg/l) and BAP (1 mg/l).

Calluses inoculation and histological sections preparation.

After one month of culture, the obtained calli were inoculated with a spore suspension prepared from a 14 days fungal culture as described by Gustine et al. (1978).

Under a laminar flow hood, the spores were delicately scraped from the fungal culture surface with a sterile spatula, then suspended in 10 ml of sterile distilled water and their concentration was adjusted to 106 spores/ml after counting with Malassez cell. About one cm in diameter callus pieces were transferred to MS medium devoid of growth regulators and then inoculated with 10µl of freshly prepared spore suspension, substituted by sterile distilled water in controls. To follow host and pathogen interaction, calli samples were taken after 24, 72, 96 hours, 7 and 14 days, and control calli were fixed in acetic ethanol (3: 1 v: v) for 24 hours and then dehydrated and embedded in paraffin. Eight µm thickness histological sections were made with a "Spencer 820" rotating microtome type. They were stained with a 0.05% toluidine blue O solution, prepared according to the method of O'Brien et al.

(1964). It is a non-specific dye which colors the pectocellulosic walls in pink, nucleic acids in blue and polyphenols in green or turquoise blue.

The slides were observed under Olympus optical microscope and the photos were taken with a "Fuji" 16 pixels digital camera.

Results

Macroscopic calluses aspect

Control (Figure 1a) and inoculated calli were checked daily to detect occurring macroscopic changes. During the first 48 hours, no change was visible to naked eye in all the genotypes tested (Figure 1b). At 72 h, hyphae appear on the calli of "Zouaoui" (Figure 1c). The mycelium developed and completely covered them on the seventh day (Figure 1d). After 14 days, the calli showed a necrotic appearance (Figure 1e). However, for the INRA 199 genotype, hyphae had a slow growth and were only visible after 96 h.

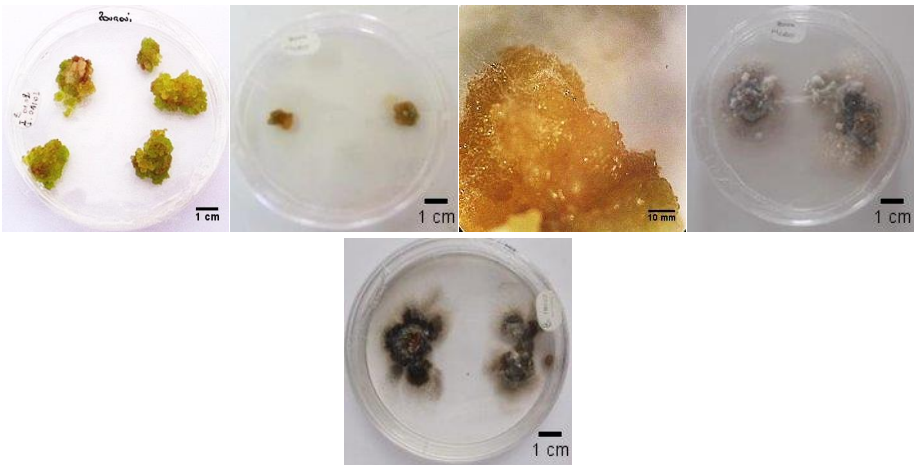

Figure 1. Zouaoui cultivar control calli (a) and inoculated after 48 h (b, c), 7 days (d) and then 14 days macroscopic appearance.

Control calli histological study

Serial sections histological investigation showed that the calli were composed of neoformed tissues consisting of a fundamental parenchymatous mass (Figure 2a). It was formed of large cells often anucleated and small cells (Figure 2b). It was also noted the presence of small isodiametric cells with strongly contrasted nucleus by the TBO (Figure2b). Their cytological characteristics were as those of meristematic cells. In addition, the presence of polyploid cells was noted (Figure 2c). In some cases, clusters of cells organized in nodules appearing on the periphery of the callus were observed (Figure 2d). These were foci of small cells with large nuclei (Figure 2e). The histological study of the different calluses also allowed the observation of newly formed tracheids (Figure 2f) present in abundance.

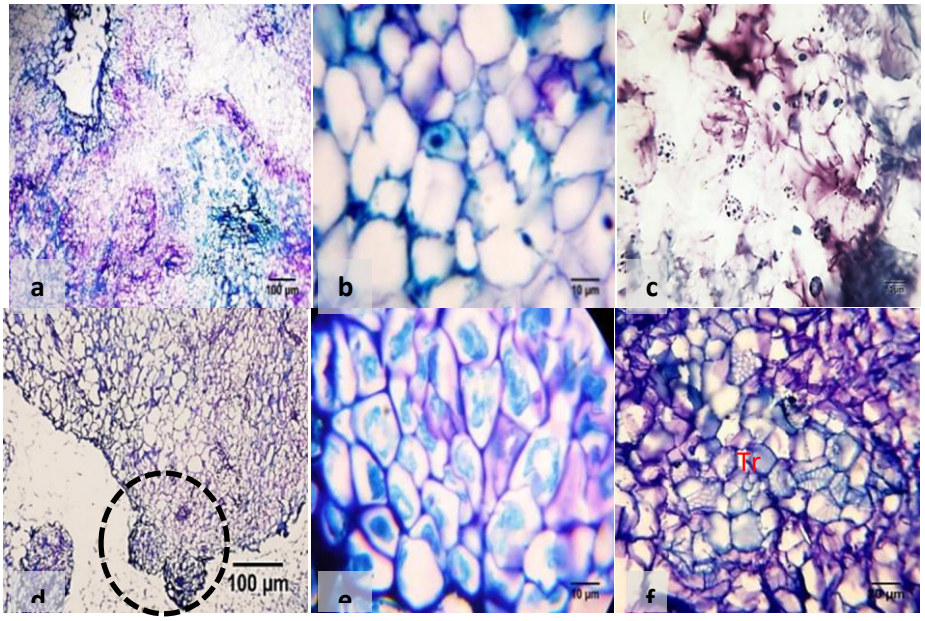

Figure 2. Control callus histological appearance. Parenchymatous cells fondamental mass (a). Big enucleated cells and small ones with a contrasted nucleus (b). Cells polyploidy (c). Cells aggregation (d) consisted of isodiametric cells with big nucleus (e) Presence of tracheids (Tr) (f)

Histological study of inoculated calli

The histological sections of the samples taken at 12 o'clock in order to demonstrate the first histological interfaces of interaction between the pathogen and the host do not reveal any modifications.

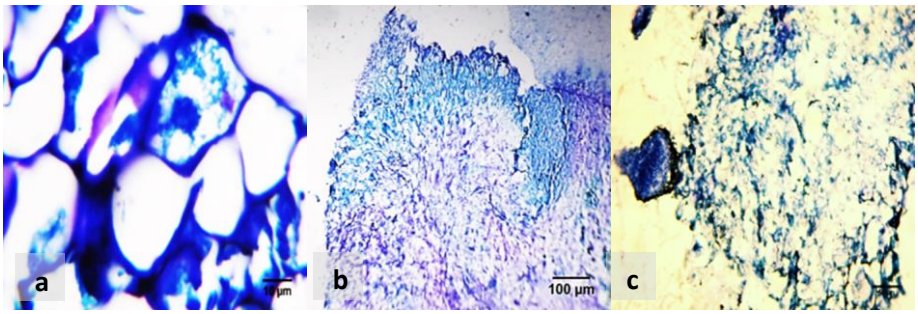

Figure 3. INRA 199 callus histological aspect after inoculation with *Ascochyta rabiei* spore suspension. Phenolic compounds accumulation (a). Boundary zone formation (b). Pycidium formation (c).

In calluses from the INRA 199 genotype, pathogen hyphae proliferated on callus surface of the after inoculation with Ascochyta rabiei spores. Several cells appeared green when stained with toluidine blue O, which reveals the presence of phenolic compounds (Figure 3a). It was noted that they formed

an area that delimited the region where the pathogen had proliferated. The growth of the mycelium increased after 72 hours (Figure 3b) but remained limited to the periphery of the callus. After 7 days of inoculation, the pathogen formed some pycnidium which released several spores on the fourteenth day (Figure 3c).

Callus from the "Zouaoui" cultivar inoculated with *Ascochyta rabiei* spores had a different profile. The callus was colonized at the periphery and deeply from the first seventy-two hours. Thus, a rapid and abundant hyphae development was noticed, and on the seventh day of infection (Figure 4a), the pathogen formed a large number of pycnidium and pycnidiospores (Figure 4b, c) released at maturity (Figure 4d) mostly unicellular and occasionally bicellular (Figure4e) in a fungal exudate (Figure 4f).

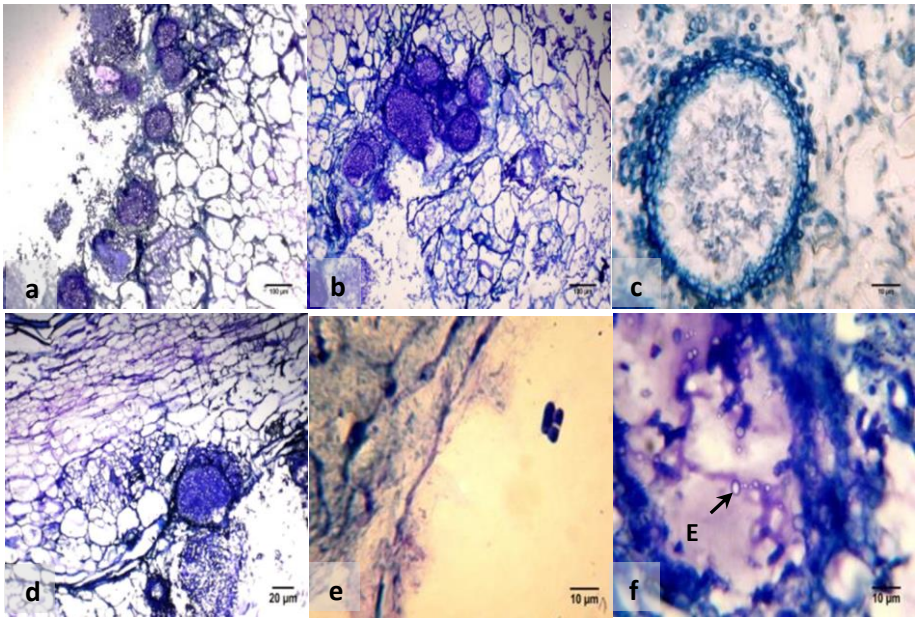

Figure 4. Zouaoui" cultivar histological aspect. Note pycnidium fast formation (a, b c) and abundant spores production (d, e) in a fungal exudat (E)

It was noted that the mycelium only proliferates in the intercellular space of the callus (Figure 5).

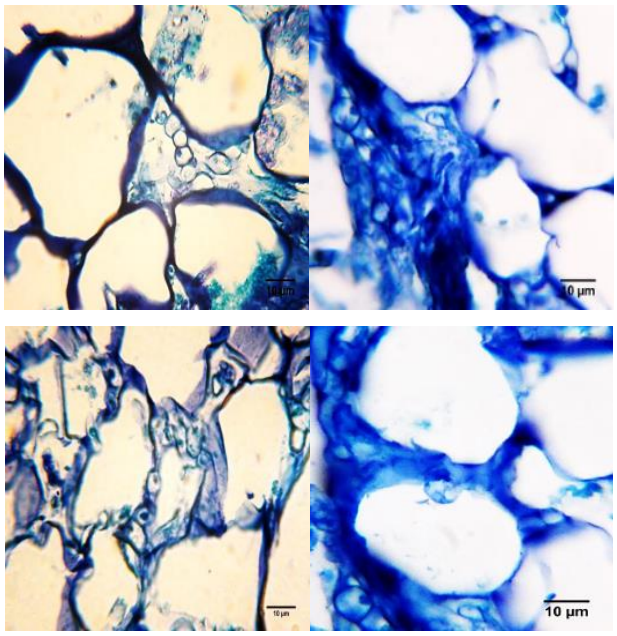

Figure 5. Hyphea presence in callus intercellular space (☆).

Discussion

Histological study showed that control calluses were formed of small parenchymal cells with contrasting nuclei, looking like meristematic cells. The culture of the explants on the media initiated a dedifferentiation of certain cells called cibled cells (Gueye et al., 2009). These undifferentiated cells proliferated in a callus heterogeneous composition (meristematic cells, parenchyma, conduction tissues, etc.). These formations were reported by numerous authors (Gautheret, 1959; Neumann et al., 2009). This study revealed the presence of newly formed tracheid. This phenomenon was reported in the case of *Cicer arietinum* (Kadiri et al., 2012) *Glycine max* var. Biloxi (Fosket and Torrey, 1969). The tracheids proliferation may be due to auxins inadequate concentration (Gautheret, 1959) and their formation involved vacuole lysis and cellular content degradation (Buchanan et al., 2015). Furthermore, a polyploidy was observed in some cases. This chromosomal abnormality was often reported in *in vitro* tissue culture (Longevialle, 1974).

INRA 199 genotype and the local cultivar "Zouaoui" reaction expression to Ascochyta rabiei was evaluated *in vitro*. Calluses derived from tissue culture were inoculated with the parasite spore suspension. This interaction was followed after 12, 24, 48, 72h, 7 days and 14 days using inoculated calli serial sections histological study after stainning with toluidine blue O.

Resistant plants selection through *in vitro* culture was a widely used procedure in phytopathology since Colins' first work on tomato in 1973 (Lebeda A and Švábová, 2010). Many studies on hosts and parasites interaction revealed that resistance and sensitivity genes can be expressed by using the whole

plant as a system or only a tissue or a cell culture (Altman and Hasegawa, 2011). According to Ingram (1967), resistance genes to *Phytophthora infestans* can easily be expressed in *Solamum tuberosum* using whole plant and tubers tissue culture. This was similarly achieved for tobacco *Nicotiana tabacum* and its parasite *Peronospora tabacina* (Trigiano et al., 1984), as well as several herbaceous and woody plants (McComb et al., 1987).

For *Cicer arietinum*/*Ascochyta rabiei* pathosystem, this work revealed that the genotypes sensitivity or the resistance expression through a system using calluses and spores was suitable. During the first 24th hours, the mycelial proliferation was relatively similar on callus from INRA 199 and "Zouaoui". A clear difference appeared only during the 48 hours following the inoculation and increased more and more over time. According to Gallão et al. (2007), it was the time required to trigger many biochemical reactions in response to infection. (Kavousi et al., 2009) work showed that in *Cicer arietinum* resistant genotypes to *Ascochyta rabiei* resistance genes expression began only 48 hours after infection. These results were in agreement with those reported by Fondevilla et al.(2015) which indicated that during the first twelve hours, only germination of some spores can be observed and more than 48 hours were required for the pathogen to penetrate host tissues. Indeed, after 48 hours, INRA 199 calluses showed a frank resistance reaction. The mycelium developed weakly in the intercellular space. Its presence was limited to the first three to five callus cells layers. This limited and weak parasite development in the callus characterized the resistance reaction in the majority of the studied pathosystems. This was the case of soybeans callus inoculated with *Phytophthora megasperma* Drechs (Holliday and Klarman, 1979), alfalfa/*Fusarium oxysporum* (Mehrotra and Aggarwal, 2013), sugar beet inoculated with *Peronospora farinosa* f.sp. *betae* (Ingram and Joachim, 1971), those of tobacco *Nicotiana tabacum* inoculated with the fungus *Phytophthora parasitica* var. *nicotinae* (DeZoeten et al., 1982) and in the case of resistant calluses of *Nicotiana tabacum*, Zea *mays* and *Triticum* sp., inoculated with *Phytophthora cinammomi* (McComb et al., 1987).

Hyphae proliferation in the intercellular space affirmed pathogen enzyme polygalacturonase (PG) and pectin methyl esterase (PME) production to degrade the pectin middle lamella cell wall main component. Moreover, the resistant genotypes callus reacted to infection also by *de novo* phenolic compounds formation whose role was well known in resistance (Clérivet et al., 1996; Macheix et al., 2005). They accumulated in the cells which constituted a discontinuous intermediate layer between the mycelium proliferation zone and the callus bottom of. This type of substance was found in all examples of tissue or cell cultures of resistant type such as rice/*Piricularia oryzea* (Uchiyama et al., 1983) *Solanum tuberosum*/*Phytophthora infestans* (Ingram, 1967), *Pennisetum glaucum*/*Sclerospora graminicola* (Upadhyaya et al., 1992), potato tubers/*Phytophthora infestans* (Doke and Tomiyama, 1980) and *Vitis venifera*/*Plasmopara viticola* (Bruno G and Sparapano, 2006). Resistant genotypes reacted by phenolic

compounds accumulation in a boundary zone cells area which stopped mycelium growth (Jang and Tainter, 1990).

On the seventh day, hyphae progression continued. They aggregated and formed a few pycnidium on resistant genotypes calli. The same observations were reported by Höhl et al. (1990), who observed the formation of a few *Ascochyta rabiei* pycnidium on chickpea resistant plant species. The fruiting body formation in resistant genotypes calli was reported in several cases of pathosystems such as *Nicotiana tabacum*/*Phytophthora parasitica* var. *nicotianae* (DeZoeten et al., 1982); *Vitis* spp./*Plasmopara viticola* (Dai et al., 1995).

Callus from the cultivar "Zouaoui" showed a total sensitivity reaction to the fungus. After 48 hours of inoculation, the mycelium rapidly proliferated through the intercellular spaces and formed pycnidium on the periphery of the callus with many spores production. Rapid callus colonization with massive fruiting body production was a common sensitivity characteristic reported in many studied pathosystems such as *Triticum aestivum* inoculated with *Neovossia indica* (Singh and Sigh, 1992).

Some cells accumulated phenolic compounds. Nevertheless, they had not guarantee any resistance to the host and this can be explained either by their little amount or by the pathogen detoxifying power (Jayakumar et al., 2005) as in *Ascochyta rabiei* virulent strains able to degrade host defence polyphenols (Kraft and Barz, 1985).

Conclusions

Chosen pathosystem plant callus/ spores allowed to follow *in vitro* the interaction between Ascochyta rabiei and its specific host *Cicer arietinum.* Calli were obtained by tissue culture from an INRA 199 genotype and a local chickpea cultivar, and then inoculated with *Ascochyta rabiei* spores. They showed two distinct histological reactions. A rapid colonization was reported of calli from "Zouaoui" compared to INRA199. Moreover, the fungus exclusively occupied the host tissues intercellular space. They reacted also by the phenolic compounds accumulation more expressed in the resistant genotypes.

REFERENCES

Altman, A. and P. M. Hasegawa (2011). Plant biotechnology and agriculture: prospects for the 21st century. Academic press.

Bruno, G. and L. Sparapano (2006). Effects of three esca-associated fungi on *Vitis vinifera* L.: I. Characterization of secondary metabolites in culture media and host responses to the pathogens in calli. Physiol. Mol. Plant Path., 69(4), 209-223.

Buchanan, B. B., W. Gruissem, K. Vickers and R. L. Jones (2015). Biochemistry and molecular biology of plants 2nd edition, John Wiley and Sons.

Clérivet, A., I. Alami, F. Breton, D. Garcia. and C. Sanier (1996). Les composés phénoliques et la résistance des plantes aux agents pathogènes. Acta Bot. Gal., 143(6), 531-538.

Dai, G. H., C. Andary, L. Mandolot-Cosson and D. Boubals (1995). Involvement of phenolic compounds in the resistance of grapevine callus to downy mildew (*Plasmopara viticola*). Eur. J. Plant Path., 101, 541-547.

DeZoeten, G. A., G. Gaad, G. T. Haberlac and J. P. Helgeson (1982). Infection of Tobacco callus by *Phytophthora paraitca* var. *nicotinae*. Phytopthology, 72, (7):743-746.

Doke, N. and K. Tomiyama (1980). Suppression of the hypersensitive response of potato tuber protoplasts to hyphal wall components by water soluble glucans isolated from *Phytophthora infestans*. Physiol. Plant Path.,16(2), 177-186.

FAO, Food and agriculture United Nations Organization (2015). (accessed 31th Nov 2015 at 5pm) http://faostat3.fao.org/

Fondevilla, S., N. Krezdorn, B. Rotter, G. Kahl, P. Winter (2015). *In planta* identification of putative pathogenicity factors from the chickpea pathogen *Ascochyta rabiei* by *de novo* transcriptome sequencing using RNA-Seq and massive analysis of cDNA ends. Front. Microbiol., 6.

Fosket, D. E. and J. G. Torrey (1969). Hormonal Control of Cell Proliferation and Xylem Differentiation in Cultured Tissues of *Glycine max* var. *biloxi*. Plant Physiol., 44(6), 871-880.

Gallão, M. I., A. L. Cortelazzo, M. P. S. Fevereiro and E. S. Brito (2007). Response to chitin in suspension-cultured *Citrus aurantium* cells. Braz. J. Plant Physiol., 19 (1), 69-76.

Gan, Y. T., K.H. M. Siddique, W. J. MacLeod, and Jayakumar (2006). Management options for minimizing the damage by Ascochyta blight (*Ascochyta rabiei*) in chickpea (*Cicer arietinum* L.). Field Crop. Res., 97, 121–134.

Gautheret, R. J. (1959). La culture des tissus végétaux: techniques et réalisations. Masson.

Gueye, B., F. Morcillo, M. Collin, D. Gargani, P. Overvoorde, F. Aberlenc-Bertossi, J. L. Verdeil (2009). Acquisition of callogenic capacity in date palm leaf tissues in response to 2, 4-D treatment. Plant Cell Tissue Organ Cult., 99 (1), 35-45.

Gupta, S., N. Nadarajan and D. S. Gupta (2014). Legumes in the Omic Era. Springer-Verlag.

Gustine, D. L., R. T. Sherwood and C. P. Vance (1978). Regulation of Phytoalexin Synthesis in Jackbean Callus Cultures. Plant Physiol., 61, 226-230.

Höhl, B., M. Pfautsch, W. Barz (1990). Histology of disease development in resistant and susceptible cultivars of chickpea (*Cicer arietinum* L.) inoculated with spores of *Ascochyta rabiei*. J. Phytopath., 129, 31-45.

Holliday, M. and W. Klarman (1979). Expression of disease reaction types in soybean callus from resistant and susceptible plants. Phytopatholology, 69, 576-578.

Ingram, D. S. (1967). The expression of R-gene Resistance to *Phytophthora infestans* in Tissue Cultures of *Solanum tuberosum*. J. Gen. Microbiol., 49 (1), 99-108.

Ingram, D. S. and I. Joachim (1971). The growth of *Peronospora farinosa* f.sp. *betae* and sugar beet callus tissues in dual culture. J. Gen. Microbiol., 69 (2), 211-220.

Jang, J. C. and F. H. Tainter (1990). Cellular response of Pine callus to infection by *Phytophtora cinnamomi*. Phytopathology, 80 (12), 1347-1352.

Jayakumar, P., Y. Gan, B. Gossen, T. Warkentin and S. Banniza (2005). Ascochyta blight of chickpea: infection and host resistance mechanisms. Periodical Ascochyta blight of chickpea. Can. J. Plant Path., 27, 499–509.

Kadiri, A., Z. Ighilhariz, L. Bouabdallah and Z. Benbayer (2012). Étude histocytochimique des cals de *Cicer arietinum* L. confrontés au mycélium *d'Ascochyta rabiei*. Synthèse: Revue des Sciences et de la Technologie, 25(1), 32-40.

Kadiri, A., Z. Ighilhariz, L. Bouabdallah and Z. Benbayer (2013). Explant source, genotype and plant growth regulators effects on chickpea (*Cicer arietinum* L.) callus induction. Int. J. Agric. Crop Sci., 5(10), 1067-1073.

Kavousi, H. R., H. Marashi and A. Bagheri (2009). Expression of phenylpropanoid pathway genes in chickpea defence against race 3 of *Ascochyta rabiei*. Plant Path. J., 8(3), 127-132.

Kraft, B. and W. Barz (1985). Degradation of the isoflavone biochanin A and its glucoside conjugates by *Ascochyta rabiei*. Appl. Environ. Microbiol., 50 (1), 45-48.

Kumar, P., M. Sangwan and N. Mehta (2010). Callogenic response of calli from chickpea genotypes on exposure to culture filtrate of *Ascochyta rabiei*. J. Mycol. Plant Path., 40 (1), 22-26.

Lebeda, A. and L. Švábová (2010). *In vitro* screening methods for assessing plant disease resistance Pages 5-45 In: Mass Screening Techniques for Selecting Crops Resistant to Diseases. International Atomic Energy Agency publications.

Longevialle, M. (1974). Première description d'un cal se prêtant à la culture indéfinie, obtenu à partir d'une Campanulacée, *Specularia speculum* L. (DC). Bulletin de la Société Botanique de France, 121, (3-4), 89-96.

Macheix, J. J., A. Fleuriet and C. Jay-Allemand (2005). Les composés phénoliques des végétaux Un exemple de métabolites secondaires d'importance économique. Presses Polytechniques et Universitaires Romandes.

McComb, J. A., J. M. Hinch and A. E. Clarke (1987). Expression of field resistance in callus tissue inoculates with *Phytophthora cinnamomi*. Phytopathology, 77(2), 346-351.

McPhee, K. E., J. Croser, B. Sarmah, S. S. Ali, D. V. Amla, P. N. Rajesh and T. J. Higgins (2007). Development of transgenics in chickpea. Pages 458-473 *in* Chickpea breeding and management. CAB International, New Delhi, India.

Mehrotra, R. and A. Aggarwal (2013). Fundamentals of Plant Pathology. Tata McGraw-Hill Education.

Murashige, T. and F. Skoog (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plantarum, 15, 443-497.

Neumann, K. H., A. Kumar and J. Imani (2009). Plant Cell and Tissue Culture- A Tool in Biotechnology. Basics and Application, Principles and Practice. Springer-Verlag.

O'Brien, T. P., N. Feder and M. E. McCully (1964). Polychromatic staining of Plant cell walls by toluidine blue O. Protoplasma, 59, 367-373.

Shah, T. M., M. Haq, B. Atta, S. Alam and H. Ali (2005). Evaluation of *Cicer* species for resistance to Ascochyta blight. Pak. J. Bot., 37 (2), 431-438.

Singh, M. and A. Singh (1992). Growth and sporulation of *Neovossia indica* in wheat callus culture. Indian Phytopath., 45(1), 113-115.

Singh, R., A. Sindhu, H. R. Singal and R. Singh (2003). Biochemical basis of resistance in chickpea (*Cicer arietinum* L.) against Fusarium wilt. Acta Phytopath. Hung., 38, 13-19.

Trigiano, R. N., C. G. Van Dyke., H. W. Spurr and D. J. Gray (1984). Infection and colonization of Tobacco callus by *Preospora tabacina*. Phytopathology, 74(3), 280-285.

Uchiyama, T., J. Sato and N. Ogasawara (1983). Lignification and qualitative changes of phenolic compounds in rice callus tissues. Agric. Biol. Chem.., 47 (1), 1-10.

Upadhyaya, G., M. B. Shivanna., H. S. Prakash and H. S. Shetty (1992). A novel approach to the establishment of dual cultures of pearl millet and *Sclerospora graminicola*. Plant Cell Tissue Organ Cult., 31 (3), 203-206.