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Optimization of *In Vitro* Culture Medium For *Cicer arietinum* and Selection of Resistant Genotypes Against Enzymes and Toxins of *Ascochyta rabiei*

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Abstract:

The contact between host plants and pathogens induces the production of phytoalexins composed of phenols, to resist against the pathogen. The aim of this study is to highlight this phenomenon using *in vitro* culture, and select resistant genotypes of chickpea (*Cicer arietinum*). Calluses of *Cicer arietinum* are exposed to the filtered filtrate (enzymes and toxins) of *Ascochyta rabiei* to demonstrate the production of phenols in the process of resistance. The calluses are produced from INRA 199 and FLIP 84-92C genotypes, as well as the Twist genotype of *C. arietinum*. The hormonal balances used are: K1 (3 mg/l 2,4-D and 1 mg/l BAP), K2 (2 mg/l 2,4-D), Ma (2 mg/l 2,4-D and 4 mg/l Kinetin), and M8 (2 mg/l 2,4-D, 4 mg/l NAA, and 1 mg/l Kinetin). The calluses are confronted with the filtered filtrate from isolates H1 and E2 of *A. rabiei*. Phenol extraction is carried out under reflux, and their quantitative estimation is performed using spectrophotometry. The results of callogenesis are influenced by genotype, organ, and hormonal balance (nature and concentration). Significant callogenesis from all four genotypes is recorded on medium K1. The results related to the interaction between *C. arietinum* calluses and enzymes and toxins of *A. rabiei* indicate that the highest quantities of phenols are produced by INRA 199 genotypes.

Keywords: *Ascochyta rabiei; Cicer arietinum*; callogenesis; enzymes; toxins; phenols.

Introduction:

To demonstrate that the most pathogen-resistant plants produce phenols, we conducted experiments on chickpeas (*Cicer arietinum*), an economically important crop which contributes to soil fertilization and can replace animal sources of proteins in poor countries (Aasim et al., 2013). This leguminous is devastated by the pathogenic fungus *Ascochyta rabiei.* Despite the use of various control methods, this pathogen continues to pose a significant threat (Jayakumar et al., 2005). Therefore, we focused on selecting resistant chickpea varieties through callus culture. Callus induction has been reported previously by several authors (Singh, 2000; Chauhan and Singh, 2002; Chauhan et al., 2003; Huda et al., 2003; Kiran et al., 2005; Naz et al., 2008a, 2008b; Rozan and Hassan, 2016).

Initially, we optimized the culture medium for callus induction in *C. arietinum.* The used culture medium was Murashige and Skoog (1962) (MS) medium. It was supplemented with the growth hormones NAA and 2,4-D as auxins, and kinetin and BAP as cytokinins, at different concentrations. Several authors reported these combinations of medium cultures for callus induction (Aasim et al., 2011; Aasim et al., 2013; Rozan and Hassan, 2016).

Subsequently, we conducted a confrontation between the callus and a filtered filtrate (containing only enzymes and toxins) of the *A. rabiei* fungus. This method has also been reported by other authors (Parkash et al., 1994; Singh et al., 2003; Sakia et al., 2006).

After confrontation, calluses produced phenols and their color turned to brown. Rouxel (1989) and Solecka (1997) also reported this result. We quantified phenols produced by calluses. Phenol extraction is carried out under reflux, and their quantitative estimation is performed by spectrophotometric determination using the Folin-Ciocalteu method (Singleton et al., 1999; Heilerova et al., 2003). This was done to determine whether the varieties producing the highest levels of phenols exhibited greater resistance to the pathogenic fungus (Kumar and Pandey, 2013; Nakabayashi et al., 2013; Gargallo-Garriga et al., 2018; Rezayian et al.,2018; Abdelaal et al., 2021; Thomas et al., 2023; Weili and Jung, 2024).

Materials and Methods:

Plant material

The plant species used in this study was *Cicer arietinum* (chickpea). The genotypes studied were: **INRA 199 (Δ1), Twist (Δ2), FLIP 84-92C (Δ3).** Seeds of the first two species were provided by the microbiology laboratory at the University of Oran. The last genotype is originated from ICADRA.

Fungal material

The fungus used in this study was *Ascochyta rabiei*. The isolates used were **H1** and **E2**. They were obtained by isolation from explants of diseased chickpea plants. The first isolate was collected from the Hammam Bouhdjar region (Algeria), and the second from Ain Témouchent (Algeria).

Methods

In vitro culture of chickpeas

Seeds were sterilized by immersion in a 5% sodium hypochlorite (NaClO) solution for 1 minute, followed by three rinses with sterile distilled water. They were then germinated in pots filled with sterile potting soil and soaked in sterile distilled water. The cultures were maintained in a greenhouse (glasshouse), and the plants were watered every three days with sterile distilled water. Explants were obtained from three-week-old plants. The most commonly used culture medium was Murashige and Skoog (1962) (MS) medium. It was supplemented with the growth hormones NAA (naphthalene acetic acid) and 2,4-D (2,4-dichlorophenoxyacetic acid) as auxins, and kinetin and BAP (6-benzylaminopurine) as cytokinins, at different concentrations. The MS medium was supplemented with 12 g of agar, 100 mg of inositol, 30 g of sucrose, and 1 ml of vitamins. The media were autoclaved at 120°C for 20 minutes after adjusting the pH to 5.8. For callus induction, stems and leaves were carefully separated, and the stems were cut into approximately 5 mm fragments without nodes. The leaves were used after sectioning the nodes. The explants were disinfected before culturing under a UV sterilized laminar flow hood according to the following protocol:

- Immersion in a 5% sodium hypochlorite solution for 3 minutes
- Immersion in a 70% ethanol solution for 30 seconds

- Rinsing 3 times with sterile distilled water
- Drying with sterile filter paper

The explants were then inoculated at a rate of 40 explants per callus induction medium. Calluses were thus initiated on MS medium supplemented with growth regulators (Table 1). These different hormones were chosen for their ability to stimulate cell growth and promote callus-type cell proliferation. The work was carried out in a UV-sterilized laminar flow hood, and the flasks were then placed in a culture chamber under a 16-hour photoperiod at 25°C.

Table 1: Hormonal Composition of Different Callus Induction Media

Growth regulators	ANA (mg/ml)	BAP (mg/ml)	KIN (mg/ml)	2-4D (mg/ml)
Ma	-	-	4	2
M8	4	-	1	2
K1	-	1	-	3
K2	-	-	-	2

Culture of the pathogen *Ascochyta rabiei*:

For the maintenance of *Ascochyta rabiei*, successive subcultures of the fungus (spaced 15 days apart) were carried out using young spores. These spores were collected from the outermost circles of colonies of different isolates. The pathogen was maintained in culture at a temperature of 22°C and a photoperiod of 12 hours. This culture was done on solid chickpea medium **(**50g flour of chickpea, 20g of glucose), which was adjusted to pH 5.8, supplemented with 12g of agar and sterilized at 120°C for 20 minutes, then poured into Petri dishes at a rate of 10 ml/dish.

Obtaining the Filtered Filtrate:

The filtered filtrate contains only the enzymes and toxins of the *Ascochyta rabiei* fungus. Thus, after 15 days of culture on solid chickpea medium, two discs of approximately 1 cm in diameter from each isolate were transferred to 100 ml of modified "Czapeck" medium. The culture was placed under a 12-hour photoperiod and at a temperature of 22°C. After 15 days, the fungal culture obtained on liquid medium was filtered through Whatman No. 1 filter paper and then through a 0.45 µm pore size Millipore filter. The mycelia were recovered, placed in

sterile glass Petri dishes, and placed in an oven at a temperature of 40°C for 8 days before being weighed. The weighing was used to determine the dry weight of the mycelium in 100 ml of "Czapeck" medium.

Confrontation:

The filtered filtrate is mixed with Murashige and Skoog (1962) culture medium without hormones and with reduced sucrose and macroelements, at a ratio of 50% filtrate and 50% MS medium. The resulting medium is poured into Petri dishes under aseptic conditions. Calluses from different genotypes are subcultured onto this confrontation medium and placed under a 16-hour photoperiod at a temperature of 22°C. Control calluses are subcultured onto MS medium mixed with 50% sterile distilled water. They are placed under the same culture conditions as the previous ones. The macroscopic evolution of the calluses is monitored daily, and a sample is taken after 72 hours to measure the total phenols produced by these calluses.

Extraction of phenols and sample preparation

The collected calluses are weighed, then ground in a mortar and boiled at 60°C in methanol (MeOH), at a rate of 10 ml of MeOH for 100 mg of callus. Two boilings are carried out. The first is done for 5 minutes and the second for 3 minutes. The obtained extracts are then filtered and the debris washed with 80% acidified MeOH (with 1% HCl). Evaporation is then carried out using a rotary evaporator at 40°C. All samples are prepared at a concentration of 0.1 g/ml (1 g of callus powder in 10 ml of MeOH), then homogenized by shaking. Storage is done in airtight tubes in the refrigerator.

Determination of phenols

The quantitative analysis of total phenols is carried out by spectrophotometric determination using the Folin-Ciocalteu method (Singleton et al., 1999; Heilerova et al., 2003). In test tubes, a quantity of 50 µl of aqueous extract (dissolved in MeOH, at a concentration of 0.1 g/ml) is added to 50 µl of MeOH (2-fold dilution) and 500 µl of Folin-Ciocalteu (Merck) (1/10 dissolved in distilled water). Agitation is carried out and the samples are placed in the dark for 5 minutes at room temperature. Another quantity of 1.5 ml of a saturated Na2CO3 solution (2%, dissolved in distilled water) is added to the mixture. A second agitation is carried out. The samples are thus placed in the dark for 1 hour at room temperature. The yellow color of the reagent turns blue. The measurements are carried out in

triplicate. The optical densities are read on a UV-Visible spectrophotometer at 760 nm using glass bowls of (volume 1 ml).

The quantification of phenols is done using a calibration curve. The latter is carried out using different concentrations of gallic acid (Fluka) dissolved in distilled water (0.025 - 0.03 - 0.035 - 0.04 - 0.045 - 0.05 - 0.055 - 0.06 mg/ml, initial concentrations). The samples are taken from a 1 mg/ml stock solution. This curve is carried out under the same operating conditions as the samples and the trend curve is **y** = 83.5**x** + 0.0766 (R2 = 0.991). The results are expressed in milligrams of gallic acid equivalent per gram of callus extract. They represent the average of 3 replicates. The blank is prepared by replacing the amount of aqueous extract with distilled water, under the same conditions.

Percentages of phenols are represented by Excel graphs. The standard deviations are less than 0,5.

Results:

Culture of the pathogen *Ascochyta rabiei*:

Isolate **H1** exhibited more cottony colonies compared to isolate **E2**. It was brown in color and grew close to the medium **(Figure 1)** with a higher growth rate than isolate **E2**. The latter, on the other hand, presented olive-green colonies. It grew close to the medium with a lower growth rate. We also observed a very clear zonation in both isolates. The two Ascochyta rabiei isolates were compared using microscopic criteria. These criteria concerned pycnidia, pycnidiospores, and mycelium. The revealed differences are represented in Table **3**.

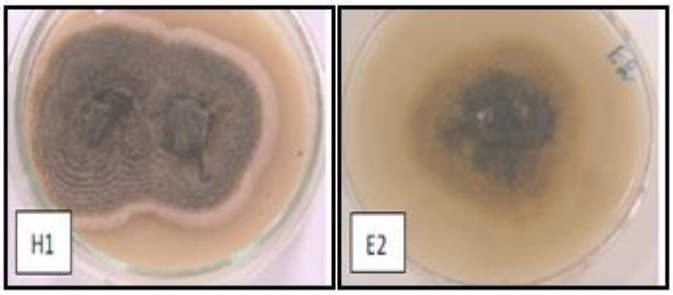

Figure 1: Macroscopic appearance of isolates H1 and E2.

In vitro culture of the host *Cicer arietinum:*

Observations made after a few days showed the beginning of modification of the explants. The leaflets curled (Figure 2D) and the stems swelled (Figure 2A, B, C). Callus formation occurred after one month (Figures 3, 4). The percentages obtained varied according to the chickpea genotypes, the types of explants, and the culture medium (Figures 5, 6, 7).

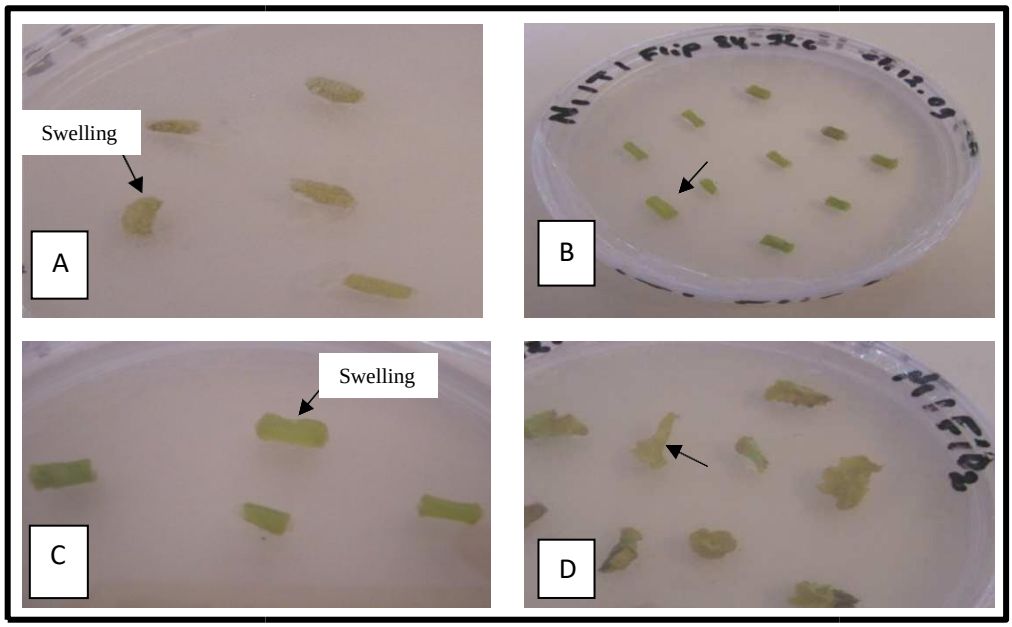

Figure 2: Initiation of hypertrophy in stems and leaves. **A** Stems of genotype Δ**1** cultivated on Ma medium exhibit noticeable swelling indicated by the arrow. **B** Stems of genotype Δ**3** grown on **K1** medium also display swelling as shown by the arrow. **C** Similar swelling is observed in the stems of genotype Δ**2** cultivated on **K1** medium. **D** Leaves of genotype Δ**2** grown on **K1** medium show a curling phenotype, as indicated by the arrow.

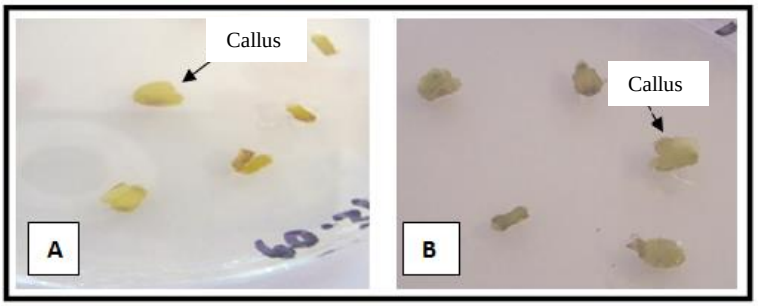

Figure 3: Calluses from stems of genotype Δ**1. A:** Calluses (arrow) from stems on **K1** medium. **B:** Calluses (arrow) from stems on **K2** medium.

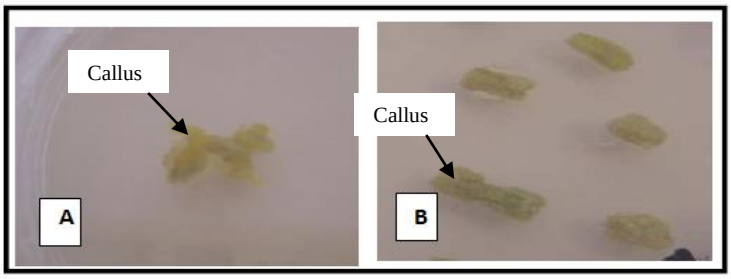

Figure 4: Calluses from leaves and stems of genotype Δ**2** on **K1** medium. **A:** Leaf callus. **B:** Stem callus.

Percentages of callus formation are represented by the following histograms.

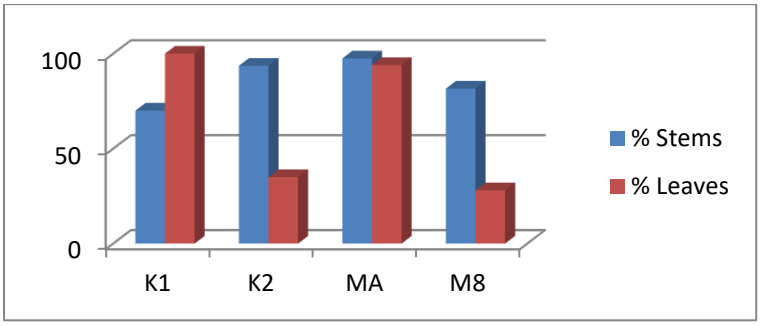

Figure 5: Histogram of callus percentages for genotype Δ**1.**

High percentages of callus formation from stems were observed on **Ma** medium (MS + 2,4-D 2mg/l + kinetin 4mg/l) and **K2** medium (MS + 2,4-D 2mg/l) (figure5). The percentage reached 97.5% on **Ma** medium, characterized by an auxin/cytokinin ratio of 1/2. On **K2** medium, the percentage was 93.61%. This indicates that callus induction can be achieved with a strong auxin alone. Other significant percentages of 81.63% and 70% were obtained on M8 medium (MS + 2,4-D 2mg/l + NAA 4mg/l + kinetin 1mg/l) and **K1** medium (MS + 2,4-D 3mg/l + BAP 1mg/l), respectively (figure 5). M8 medium combines two auxins with one cytokinin, while K1 medium is a combination of only two hormones (one auxin: 2,4-D and one cytokinin: BAP). These results suggest that the combination of two auxins and one cytokinin promotes callus formation. Leaves exhibited varying percentages of callus formation (Figure 5). A maximum value of 100% callus formation was observed on **K1** medium. On this medium, kinetin was replaced by BAP. Another significant value of 94% was observed on Ma medium. These results indicate that the substitution of kinetin with BAP promotes callus formation.

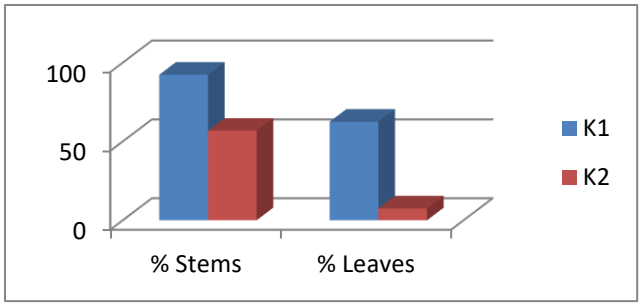

Figure 6: Histogram of callus percentages for genotype Δ**2.**

Figure 6 shows that the percentage of stem calluses for genotype Δ**2** was 56.75% on K1 medium and double this value (92.3%) on **K2** medium. Leaf explants produced a significant percentage of calluses (62.5%) on the same medium (**K1**). A relatively low rate of leaf calluses (7.5%) was observed on **K2** medium, which contains only an auxin (2,4-D). The absence of cytokinin seems to affect callus formation in leaves but not in stems.

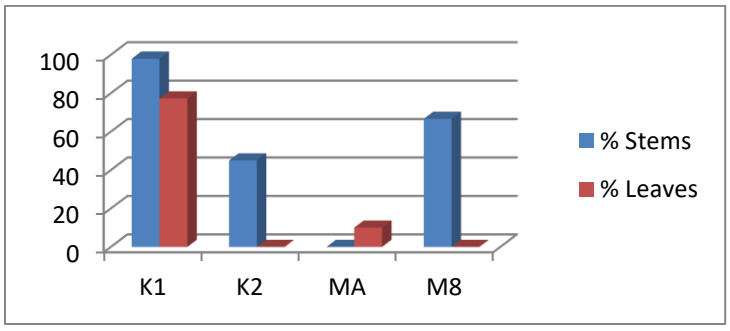

Figure 7: Histogram of callus percentages for genotype Δ**3.**

Figure 7 shows that the highest callus formation percentages, 97.96% and 77.27% for stems and leaves of Δ**3** respectively, were obtained on **K1** medium. For stems, percentages close to half were observed on **M8** and **K2** media.

Confrontation:

The evolution of confronted calluses was monitored and compared to that of control calluses. No changes were observed within the first 24 hours of confrontation. After 72 hours, browning of calluses was observed in all genotypes, indicating an accumulation of phenols. No changes were reported in the controls (Figures 8, 9). After eight days of confrontation, all calluses had turned dark brown compared to the controls.

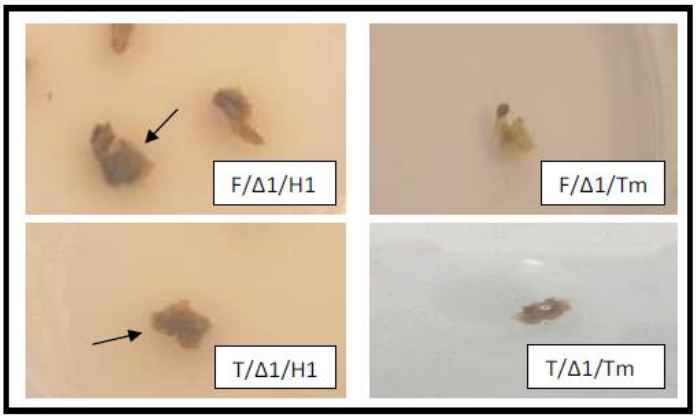

Figure 8: Appearance of calluses from genotype Δ**1** and their controls after 72 hours of confrontation. **F**: leaves, **T**: stems, Δ**1**: genotype INRA 199, **H1:** isolate **H1** of *Ascochyta rabiei.* **Tm**: control. **Arrows**: browning.

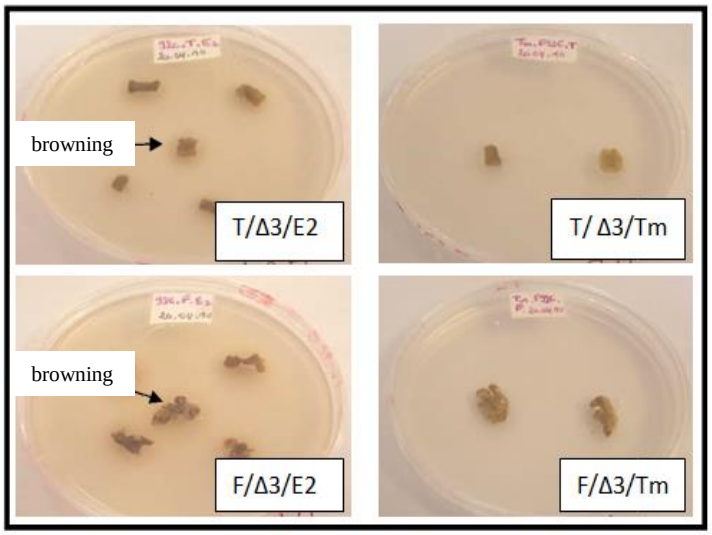

Figure 9: Appearance of calluses from genotype Δ**3** and their controls after 72 hours of confrontation. **F**: leaves, **T**: stems, Δ**3**: genotype FLIP 84-92C, **E2**: isolate E2 of *Ascochyta rabiei*. **Tm**: control. **Arrows**: browning.

Total Phenol Content:

The phenol content was obtained from a calibration curve established with increasing concentrations of gallic acid (Figure 10). It is expressed in milligrams of gallic acid per gram of extract (mg GA/g). $Y = 83.5X + 0.076$ ($R^2 = 0.991$). Figure 11 shows the different values obtained.

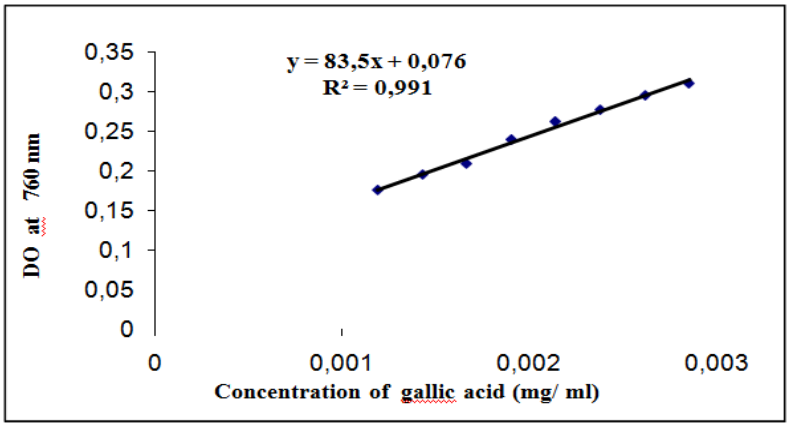

Figure 10: Calibration curve of gallic acid

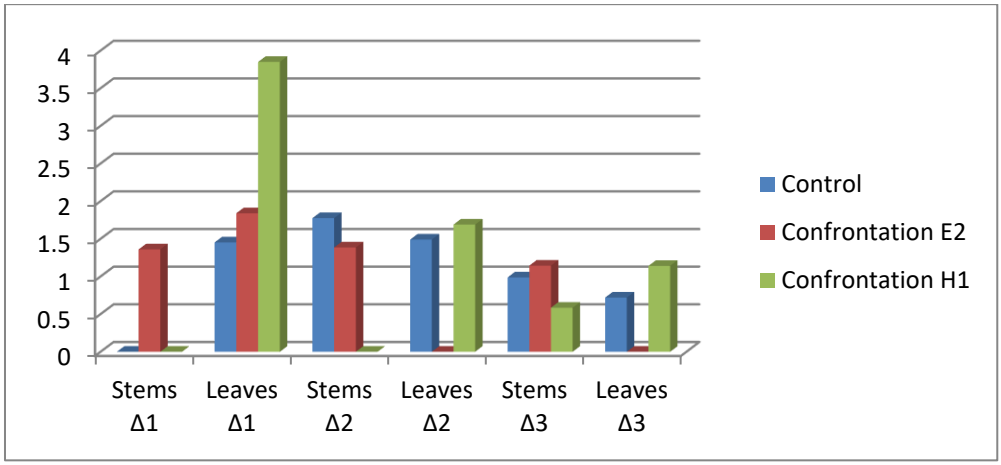

Figure 11: Phenol content in calluses tissues (stems and leaves) of chickpea genotypes (**Δ1, Δ2, Δ3**) when confronted with two isolates (**H1** and **E2**) of *Ascochyta rabiei.*

Control calluses produced phenols with concentrations ranging from 0.53 mg/g to 0.72 mg/g. The quantities of phenols produced by the controls were lower than those produced by calluses treated with the *Ascochyta rabiei* filtrate. However, there were two instances where phenol content in the controls was slightly higher than that in the treated calluses. Stem callus controls of genotype **Δ2** showed a phenol content of 1.77 mg/g, while treated calluses yielded a value of 1.38 mg/g. Similarly, stem calluses controls of genotype **Δ3** produced 0.98 mg/g of phenols, which was higher than that produced by stem calluses confronted with isolate **H1** (0.58 mg/g). The maximum values were obtained in genotype **Δ1**, specifically in leaf explants.

A value of 3.85 mg/g was observed in explants confronted with isolate **H1**. This value is double that of explants confronted with isolate **E2** (1.84 mg/g).

Callus tissues from the stems of genotype Δ**2**, when confronted with isolate **E2**, produced the same amount of phenols (1.38 mg/g) as those from the stems of genotype Δ**1** under the same treatment. Similarly, control leaf calluses of both genotypes produced the same amount of phenols (1.49 mg/g). Leaf calluses from genotype Δ**2** exposed to isolate **H1** produced a lower quantity (1.68 mg/g) compared to those from genotype Δ**1** (3.85 mg/g).

Calluses originating from organs of genotype Δ**3** exhibited the lowest phenol content. Stem calluses confronted with isolate **E2** produced an estimated quantity of 1.14 mg/g. This value is double that revealed by the confrontation with isolate **H1** (0.58 mg/g).

The obtained results indicate that genotype Δ1 produced the maximum quantities of phenols. Genotype Δ**2** produced lower quantities of phenols, while calluses from genotype Δ**3** yielded the lowest levels.

Discussion of Results:

In Vitro Culture:

Our results indicate that callus induction occurred after 10 to 14 days. All authors obtained the onset of callus formation within the same timeframe. Callus formation in *Cicer arietinum* is influenced by hormonal combinations (nature and concentrations), the type of explant (stems or leaves), and the genotype (Riazuddin et al., 1988; Barna and Wakhulu, 1994). Our study yielded high callus induction rates on MS medium supplemented with auxins and cytokinins. Morjane and Harrabi (1993), Kumar et al. (1994), Aasim et al. (2011), Aasim et al. (2013), Rozan and Hassan (2016) also reported that MS medium containing cytokinins combined with auxins induced callus formation of *Cicer arietinum* .

A significant amount of callus was initiated in both leaf and stem explants in the presence of 2,4-D (3 mg/L) and BAP (1 mg/L) with an auxin/cytokinin ratio of 3. Huda et al. (2003a) and Huda et al. (2003b) obtained a very high callus induction rate with the same hormonal combination using cotyledons of *Cicer arietinum*,.

Sagare et al. (1993) obtained good callus induction rates from cotyledonary segments of *Cicer arietinum* (C235) with the same hormonal composition but using different

concentrations (3 mg/L of 2,4-D and 0.1 mg/L of BAP). Naz et al. (2008a) also observed high percentages of callus from cotyledons and leaves of chickpea (genotypes: CV, CM72) on this hormonal combination. Rozan and Hassan (2016) also obtained a very high callus induction rate with the same hormonal combination using cotyledons of *Cicer arietinum*.

Replacing 2,4-D with NAA in M8 medium, favored callus production from internodes and leaf explants. Barna and Wakhulu (1994), Huda et al. (2000), Houda et al. (2001), Arora and Chawla (2005) also initiated callus formation from chickpea stem internodes (genotypes: Pusa 256 and PG 186) by replacing 2,4-D with NAA. They obtained calluses on MS medium supplemented with NAA (3 mg/L) and BAP (3 mg/L).

Significant callus formation from stems of the tested genotypes was reported in the presence of 2,4-D alone. This finding is in agreement with results of Singh et al. (1999) and Huda et al. (2003a) who obtained calluses in *Cicer arietinum* using 2 mg/L of 2,4-D without cytokinins. The use of 2,4-D alone is better than NAA (Barna and Wakhulu, 1993; Barna and Wakhulu, 1994). Morjane and Harrabi (1993) observed callus formation on MS medium supplemented with 2,4-D alone, they reported that root explants of *Cicer arietinum* (cv. Amdoun) produced calluses on MS medium containing 2,4-D (1 mg/L) and (2 mg/L). It is noteworthy that the use of auxin alone can initiate callus formation in *Cicer arietinum* leaf explants (Aasim et al., 2011; Aasim et al., 2013).

Similar to the finding of Naz et al. (2008b) who tested cotyledons and leaves of chikpea, the combination of three hormones, such as two auxins and one cytokinin (2,4-D, NAA, kinetin) in M8 medium, allowed for moderate callus formation, except that kinetin was replaced by BAP in their culture medium.

Confrontation:

Plant-pathogen interaction induces a hypersensitive response, characterized by pigmented necrosis. This reaction is due to accumulation of phenols. The synthesis of these compounds occurs following stress (Rouxel, 1989; Solecka, 1997; Tomas et al., 2023; Tan and Goren, 2024) and their accumulation limits pathogen growth. In resistant plants, defense is based on the accumulation of phenols at the infection site (Chérif et al., 2007; Khan et al., 2010; Nakabayashi et al., 2013; Gargallo-garriga et al., 2018; Tan and Goren, 2024).

Observations show a browning of calluses, corresponding to production of phenols after the confrontation with filtered filtrate of the pathogen *Ascochyta rabiei*. Confronted calluses

produced more phenols than untreated calluses. This production varies according the genotype (resistant or tolerant) and the isolate (H1 and E2). Maximum values of phenols were obtained with INRA199 (resistant genotype). These results are in accordance with ones of Parkash et al. (1994), Vogelsang et al. (1994), Singh et al. (2003).

High production of phenols by resistant genotypes of *Cicer arietinum* was observed after confronting calluses of chickpea with filtered filtrate of *Ascochyta rabiei,* and these results are in accordance with ones of Daniel et al. (1990) who confronted chickpea cell suspensions with a polysaccharide from *Ascochyta rabiei*. Hinderer et al. (1987) also reported an accumulation of phytoalexins in *Cicer arietinum* cells. This accumulation occurred after confronting chickpea cell suspensions with an enzymatic preparation of hydrolases.

Singh et al. (2003) confronted *Cicer arietinum* calluses with the filtrate of *Fusarium oxysporum f.sp. ciceri*. They estimated the quantity of phenols in the calluses. Resistant genotypes produced maximum values. Parkash et al. (1994) also confronted *Cicer arietinum* calluses with the culture filtrate of *Fusarium oxysporum f.sp. ciceri.* Their results demonstrated that the phenol content was higher in resistant genotypes.

Khan et al. (2005) inoculated *Cicer arietinum* roots with the culture filtrate of *Fusarium oxysporum f.sp. ciceri*. The quantity of phenols was estimated before and after treatment. It revealed an increase in phenol production following treatment. It is also worth noting that resistant genotypes produced higher quantities compared to susceptible ones.

Sakia et al. (2006) studied the interactions between *Cicer arietinum* and *Fusarium f.sp. ciceri* and *Cicer arietinum* and *Macrophomina phaseolina*. Their work involved treating seeds with pathogen cell wall proteins. A phenol dosage was then performed. They reported an increase in phenol synthesis after confrontation.

The study by Khan et al. (2010) on the interaction between a chickpea cell suspension and *Candida albicans* filtrate demonstrated the production of phenols in the context of resistance.

Vogelsang et al. (1994) brought *Ascochyta rabiei* filtrate into contact with a chickpea cell suspension. They observed browning of cells in the resistant genotype. The browning was due to the production of isoflavonoids. A toluidine blue test indicated the accumulation of phenolic compounds in the cell walls of resistant genotypes. These results were not observed in susceptible genotypes.

Conclusion:

To select resistant genotypes of chickpea via biotechnology, we carry out an *in vitro* culture, then a confrontation of the obtained calluses to the filtered filtrate of the fungus *Ascochyta rabiei*, which contains only enzymes and toxins.

Significant calluses formation was recorded on MS medium supplemented with 2,4-D and BAP. Our results also show that phenols are produced during the interaction between *Cicer arietinum* calluses and the filtered filtrate (enzymes and toxins) of *Ascochyta rabiei*. The quantities produced by calluses of the resistant genotype INRA 199 were the highest in the presence of isolates H1 and E2. Calluses of the susceptible genotype Twist exhibited fewer phenols than the INRA 199 genotype when confronted with both isolates.

This study has allowed us to find a culture medium composition favorable for the *in vitro* culture of chickpea and probably demonstrated the involvement of phenols in the resistance phenomenon of *Cicer arietinum* calluses to enzymes and toxins of the pathogen *Ascochyta rabiei*.

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