

## **Crown Gall Disease in Moroccan Almond Trees: Tumorigenic Bacteria and Sustainable Management through Biological Control**

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## Abstract

Crown gall is a globally recognized bacterial disease caused by *Agrobacterium tumefaciens*. This bacterium is characterized by its potential to infect a wide range of plants, specifically fruit trees. In Morocco, almond trees (*Prunus amygdalus*) are among the most heavily affected species by this disease. In our study, all tested bacteria were isolated in the laboratory from infected almond samples exhibiting tumors at the crown and root of the sampled trees collected from northwestern of Morocco. Biochemical, pathogenicity and molecular identification were performed to identify the causal agent of almond crown gall. Molecular identification was done using a duplex PCR (A/C9 and A/E9) targeting *virD2* gene located in the pTi plasmid, which is the oncogenic element of the bacterium. The results showed that 12 from 20 isolates were identified as tumorigenic *A. tumefaciens* with the presence of pTi. Moreover, these isolates were found to exhibit tumorigenic properties both *in vitro* and *in vivo* when tested on indicator plants, specifically tomato and carrot discs. The efficacy of agrocin synthesized by *Agrobacterium radiobacter* strain K1026, a biopesticide known as NoGall, was tested *in vitro* and *in planta* against tumorigenic strains of *A. tumefaciens*. Our results revealed a significant inhibitory effect *in vitro*, with inhibition percentages ranging from 29.5% to 38.9%. Additionally, we observed a complete reduction in tumor growth at the inoculation sites on indicator plants, with a 100% reduction percentage.

**Keywords:** Almond tree, crown gall, *Agrobacterium tumefaciens*, *Agrobacterium radiobacter*, K1026, agrocin, biological control.

## La galle du collet de l'amandier au Maroc : Bactéries Tumorigènes et Gestion Durable par le Contrôle Biologique

### Résumé

La galle du collet est une maladie bactérienne causée par *Agrobacterium tumefaciens*. Cette bactérie se caractérise par son potentiel à infecter une large gamme de plantes et plus particulièrement les arbres fruitiers. Au Maroc, les amandiers (*Prunus amygdalus*) sont parmi les espèces les plus touchées par cette maladie. Dans notre étude, toutes les bactéries testées ont été isolées en laboratoire à partir d'échantillons d'amandiers infectés présentant des tumeurs dans le collet et le système racinaire des arbres échantillonnés dans le nord-ouest du Maroc. L'identification biochimique, bactériologique et moléculaire a été effectuée afin d'identifier l'agent causal de la galle du collet de l'amandier. L'identification moléculaire a été réalisée à l'aide d'une PCR duplex (amorces : A/C9 et A/E9) ciblant le gène *virD2* situé dans le plasmide pTi, qui est l'élément oncogène de la bactérie. Les résultats ont montré que 12 sur 20 isolats étaient identifiés comme des *A. tumefaciens* tumorigènes avec la présence de pTi. De plus, les isolats identifiés se sont avérés tumorigènes *in vitro* et *in vivo* sur les plantes indicatrices (plantes de tomate et les tranches de carotte). L'efficacité de l'agrocine synthétisée par la souche K1026 d'*Agrobacterium radiobacter*, un biopesticide connu sous le nom de NoGall, a été testée *in vitro* et *in planta* contre des souches tumorigènes d'*A. tumefaciens*. Les résultats obtenus ont montré un effet inhibiteur significatif *in vitro* avec un pourcentage d'inhibition compris entre 29,5 et 38,9% et une réduction de la croissance tumorale aux sites d'inoculation sur les plantes indicatrices, avec un pourcentage de réduction de 100%.

**Mots-clefs** : Amandier, galle du collet, *Agrobacterium tumefaciens*, *Agrobacterium radiobacter*, K1026, agrocin, lutte biologique.

## مرض التدرن التاجي لشجرة اللوز: التوصيف الجزيئي للبكتيريا المسرطنة ودراسة فعالية المكافحة البيولوجية

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### ملخص

التدرن التاجي هو مرض بكتيري تسببه البكتيريا الممرضة أكروباكتيريوم توميفاسيان. تتميز هذه البكتيريا بإمكانية إصابة مجموعة واسعة من النباتات وخاصة أشجار الفاكهة. في المغرب، تعد أشجار اللوز (برونوس اميغدالوس) من بين الأنواع الأكثر تضرراً من هذا المرض. في دراستنا التالية، تم عزل جميع البكتيريا المدروسة في المختبر من عينات من أشجار اللوز المصابة تظهر أورامًا في تاج الشجرة والجذور لعينات أشجار في شمال غرب المغرب. تم إجراء التعرف البيوكيميائي والبكتريولوجي والجزيئي لتحديد العامل المسبب لمرض التدرن التاجي لشجرة اللوز. تم إجراء التحديد الجزيئي باستخدام *PCR* مزدوج (بادئات: *E9 / A* و *C9 / A*) لاستهداف جين فيروس *2 (virD2)* الموجود في بطي بلاسميد (*pTi*) ، وهو العنصر المسرطن للبكتيريا. أظهرت النتائج أن غالبية عزلاتنا قد تم تحديدها على أنها بكتيريا ممرضة مع وجود الجين فيروس *2*. بالإضافة إلى ذلك، ثبت أن العزلات التي تم التعرف عليها قادرة على تكوين أورامًا في المختبر وفي الجسم الحي على النباتات المؤشر (نباتات الطماطم وشرايح الجزر). تم اختبار فعالية الأجروسين المُصنَّع بواسطة سلالة أكروباكتيريوم راديوباكتير *ك1026 (K1026)*، وهو مبيد حيوي معروف باسم نوغال (*NoGall*)، في المختبر وفي النباتات ضد السلالات الممرضة. أظهرت النتائج التي تم الحصول عليها وجود تأثير مثبت معنوي في المختبر بنسبة تثبيط تراوحت بين 29.5 و 38.9٪ وانخفاض في نمو الورم في مواقع التلقيح على النباتات المؤشر بنسبة 100٪.

**الكلمات المفتاحية:** شجرة اللوز، التدرن التاجي، أكروباكتيريوم توميفاسيان، أكروباكتيريوم راديوباكتير، الأجروسين، ك1026، المكافحة البيولوجية

## Introduction

The almond industry holds a prominent position in Moroccan agriculture. This species has demonstrated great resilience in the face of drought-related problems caused by climate change. It has experienced significant growth in terms of area and production, thanks to various research and development programs initiated previously by the Green Morocco Plan and currently by the Generation Green program (MAPMDREF, 2022).

However, like other fruit trees, almond cultivation is sensitive to various abiotic and biotic stresses, particularly those caused by phytopathogenic agents, pests, and weeds that affect various organs (Benjma et al. 1996; Benjama, 2005). In this regard, crown gall disease of almond, caused by *Agrobacterium tumefaciens* (Smith and Townsend, 1907), is one of the most common and widely distributed bacterial diseases worldwide (Benjama et al. 2004). It is considered one of the most destructive bacteria, causing significant financial losses: 10% of the nursery plant material which equals to 112 M\$ in one year and 23M\$ of estimated damages of the crown gall in one year in USA (Krimi et al. 2002). It was first reported in Morocco in 1960 in the Fez-Meknes region (Benjama et al. 2002).

*Agrobacterium tumefaciens* is a rhizospheric bacterium that has the ability to transform plant cells if these bacteria acquire the Ti plasmid (Tumor inducing) through conjugation, which is essential for the bacterium's pathogenicity (Thomashow et al., 1984). The infection mechanism of *A. tumefaciens* is a transgenic phenomenon in which the bacterium genetically transforms the plant cell by introducing a portion of its Ti plasmid, known as T-DNA (Transfer DNA), into the plant cell's genome. This transformation is manifested, on one hand, by the production of phytohormones (auxins and cytokinins) that induce hierarchical cell division leading to tumor formation, and, on the other hand, by the synthesis of opines, which are compounds that serve as a source of carbon, nitrogen, and energy for bacterial growth (Burr, 2004). The tumors formed on the tree's collar block the vascular tissues, affecting the circulation of water and mineral elements, thereby disrupting the growth of the almond tree. This ultimately leads to decreased productivity, stunted growth, and, in severe cases, death of the tree (Burr et al., 1998).

Therefore, it would be interesting to develop early and reliable diagnostics to anticipate appropriate phytosanitary treatments capable of reducing or completely inhibiting the proliferation of this disease. To date, all tested treatments (cultural and chemical techniques) have been ineffective against crown gall (Megateli, 2012; Tolba and Soliman, 2014; Habbadi et al., 2017). In general, the management of the disease is based on cultural prevention techniques. Therefore, it is crucial to explore alternative solutions to control this bacterium, including biological control methods that utilize phytosanitary products containing active ingredients derived from organisms (plants), microorganisms (bacteria, yeasts, fungi and viruses) or even natural substances (essential oils, plant extracts). Indeed, these treatments have demonstrated significantly greater protective efficacy compared to chemical products (Chen et al., 2007). Embracing biological control methods against this pathogen holds promise, as it safeguards the environment from the excessive use of chemical agents while ensuring higher yields of nutritionally sound produce in accordance with health standards (Spadaro and Gullino, 2004).

The use of non-tumorigenic strain *Agrobacterium* constitutes one of the biological control methods employed against tumorigenic strains. The *Agrobacterium radiobacter* K84 (Kerr and Htay, 1974) stands as the pioneering model used to control crown gall since its unique properties were discovered by Dr. Allen Kerr in 1970s in Australia. This particular strain was selected for the development of an effective universal biopesticide (current trade name: Dygall & NoGall), which clearly reduces the colonization of tissues by the pathogen (Bazzi et al., 1999). This biological control agent acts by antibiosis by producing an antibiotic which is agrocin thanks to the presence of a specific plasmid (pAg K84). The antibiotic enters the target cells by way of a permease, facilitating the passage of the two opines, namely nopaline and agropine, across the membrane and into the pathogenic bacterium (Kerr and Htay, 1974).

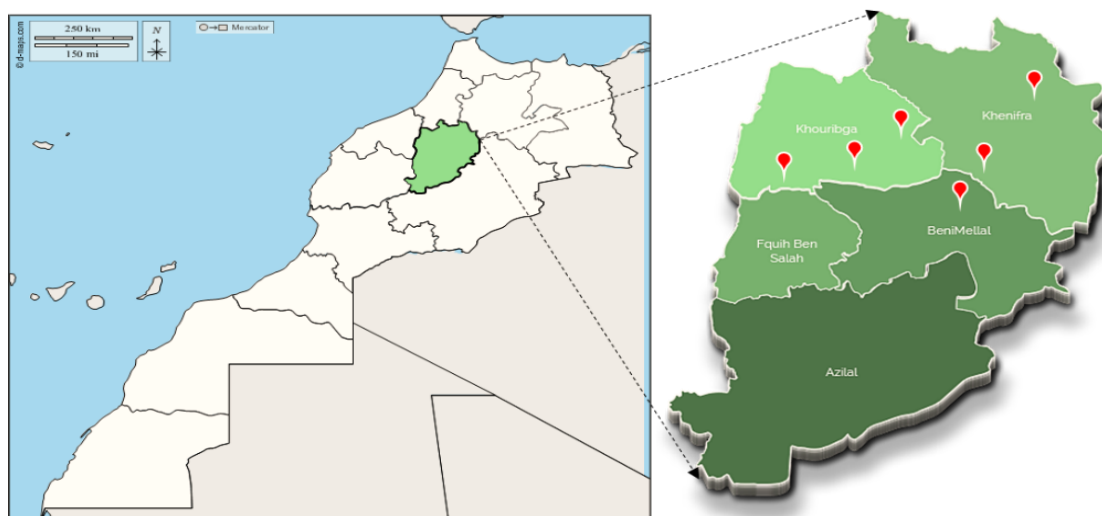
The present study, conducted within the framework of this approach, aims to: (1) characterize the molecular aspect of new isolates of *A. tumefaciens* and study their pathogenicity on *in vitro* and *in planta* indicator plants, and (2) evaluate the efficacy of the biological control agent *A. radiobacter* strain K1026 both *in vitro* and *in planta*.

## Materials and Methods

### Sample Collection

For the characterization of *A. tumefaciens* populations responsible for almond crown gall disease, systematic surveys were conducted from 15 April to the end of July 2021 in several almond productions in north western of Morocco (Figure 1). The visited plots were randomly selected without any defined criteria. The surveys were carried out by completing a technical form containing information such as GPS coordinates, address, varieties, age, and cultural practices.

Sampling was performed from newly developed tumors on the collar of almond trees and from soil in the rhizosphere of diseased trees. The collected samples were placed in plastic bags with a specific code, kept cool in an icebox, and transported to the laboratory for isolation and analysis.



**Figure 1.** Sampling site of the study.

### **Isolation of bacteria**

For tumor samples, isolation was performed using the method described by Moore et al. (1988 & 2001). In this procedure, tumour tissues were washed under running water and necrotic tissues were removed; tumour surface was rinsed with 70% ethanol and cut into small pieces under axenic conditions. These pieces were crushed with a micropestle in 2 mL of sterile distilled water and let to macerate for at least 30 min. From the aqueous phase, serial dilutions were made and 20 µL of suspension were streaked on MG medium and incubated on 28°C during 48 hr. Single colonies obtained with morphological resemblance to *Agrobacterium* spp. were purified on MG supplemented with potassium tellurite (200 mg/l) (MG+T) (Brisbane and Kerr 1983; Mougel et al. 2001; Shams et al. 2012).

After growth, typical *Agrobacterium* colonies (e.g. circular glistening morphology plus a characteristic black colour with a metallic shine due to incorporation of tellurite) were picked and suspended separately in 100 µL sterile distilled water and incubated overnight at 28°C. A final purification was made on MG medium. Cultures were stored at 4°C on MG medium for short-term maintenance and frozen at -80°C in nutrient broth with 25% glycerol for long-term preservation.

### **Duplex PCR Amplification**

DNA preparation for PCR amplification was performed as described previously (Campillo et al. 2012). Briefly, cells from a single colony were resuspended in 10 µL of 20 mmol/L NaOH and incubated at 37°C for 5 min. Three microliters of this suspension were used for the duplex PCR in reaction mixtures of 20 µL. The primers (A/C9 and A/E9) used for PCR amplification in the present study are listed in Table 1. These primers targeting the *virD2* genes located in the transfer DNA (T-DNA) fragment of the Ti plasmid (tumor-inducing plasmid) (Haas et al. 1995).

Standard PCR was carried out in 50 µL reaction volume containing 4 µL of Bioline buffer (5 mM dNTPs and 15 mM MgCl<sub>2</sub>), 10.8 µL of sterile ultrapure water (SUPW), 0.5 µL of each primer, 0.2 µL of Taq DNA polymerase (Bioline), and 3 µL of lysed cells. The PCR was performed using the following program: an initial denaturation step at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 1 minute, annealing at 57°C for 1 minute, and extension at 72°C for 1 minute, followed by an additional extension at 72°C for 3 minutes. The reference strain *A. tumefaciens* C58 (Kerstens et al., 1973) was used as a positive control. Electrophoresis was performed on a 2% agarose gel (2 g agarose in 100 mL of TBE buffer) stained with ethidium bromide. Electrophoresis was conducted for 15 minutes at 200 V. The results were visualized under ultraviolet (UV) light.

**Table 1.** PCR primers used in this study.

Gene	Primer	Primer sequence	Reference
<i>virD2</i>	A	ATGCCCGATCGAGCTCAAGT	Haas et al., 1995
	C9	TCGTCTGGCTGACTTTCGTCATAA	
	E9	CCTGACCCAAACATCTCGGCTGCCCA	

## Pathogenicity Test

To validate the molecular data, the pathogenicity of the isolates was assessed *in planta* using indicator plants (tomato and kalanchoe) and *in vitro* on carrot slices.

The tumour-forming ability of isolates was determined by inoculating wounded stems of 3-week-old seedlings of tomato (*Solanum lycopersicum* L.) and Kalanchoe (*Kalanchoe daigremontiana*). Briefly, the indicator plants were wounded at the top of the stem just under the second leaf, and were inoculated with 10  $\mu$ L ( $10^8$  CFU/mL) of an overnight bacterial culture. Inoculated plants were maintained in greenhouse at 22°C, with a relative humidity between 50 and 80% and a photoperiod of 16 h/8 h light/dark. The presence or absence of tumour was visualized 3 weeks post infection. *A. tumefaciens* C58 was used as positive controls.

For the pathogenicity test on carrot slices, intact and disinfected carrots with 10% bleach and washed two times with sterile distilled water (SDW), followed by drying. The carrots were cut into rounds and placed in a Petri dish with a filter paper soaked with SDW to maintain a humidity level of around 80%. 10  $\mu$ L of the bacterial suspension of the tested isolate at a concentration of  $10^8$  CFU/mL was deposited on the root vessels of the carrot slice, specifically on the lower side facing the ground. *A. tumefaciens* C58 was used as positive controls and SDW as a negative control. The slices were then incubated for 10 days at  $27 \pm 2^\circ\text{C}$ .

## Biological control in vitro

The effectiveness of strain K1026 (Jones and Kerr, 1989) was evaluated by testing the efficiency of the antibiotic substance secreted by this strain, which is agrocin. The extraction of this substance was performed by confronting strain K1026 and the reference strain *A. tumefaciens* C58 in liquid YPG medium, followed by the spot test. To do this, an equal mixture of the two bacterial suspensions of the two strains (K1026 and C58) at an equivalent concentration of  $10^8$  CFU/mL was prepared. The mixture was incubated under agitation for 24 hr at a temperature of  $27 \pm 1^\circ\text{C}$ . After incubation, centrifugation was performed for 7 minutes at 8000 g, followed by filtration using a 0.2  $\mu$ m filter to recover the supernatant containing agrocin.

The evaluation of agrocin efficacy is performed on tumorigenic strains of *A. tumefaciens* isolated from almond trees using an aromatogram test. A bacterial suspension of  $10^8$  CFU/mL of each *A. tumefaciens* isolate was prepared from an overnight bacterial culture. One hundred microliters of the bacterial suspension were inoculated using the flooding method on YPG medium. The Petri dishes (90 mm) were placed under a laminar flow hood to dry for 15 to 20 minutes. On the surface and in the middle of each dish, a sterile filter paper discs (5 mm diameter) was soaked with 2  $\mu$ L of agrocin solution. The filter soaked with 2  $\mu$ L of SDW was served as negative control and filter impregnate with streptomycin (32mg/L) was served as positive control. The dishes are incubated at  $27 \pm 1^\circ\text{C}$  for 24 to 48 hr. After incubation, the inhibition zone around each disc was measured and the percentage inhibition was calculated considering that no growth corresponds to 100% inhibition.



## In planta Confrontation Test

Young tomato plants at the 3-4 leaf stage (2 to 3 weeks old) are selected for inoculation. Using a sterile scalpel, a wound is made on the stem of the tomato plants, and then 10  $\mu\text{L}$  of the bacterial suspension of strain K1026 ( $10^8$  CFU/mL), prepared from an overnight bacterial culture, is injected first at the site of the wound using a micropipette. After a duration of 24 hr, 10  $\mu\text{L}$  of the bacterial suspension of the pathogenic *A. tumefaciens* ( $10^8$  CFU/mL) were inoculated at the wound site. Untreated tomato plants inoculated with strain C58 or SDW are considered as positive and negative controls, respectively. The inoculated plants are kept in a greenhouse at  $27 \pm 1^\circ\text{C}$  for 15 to 20 days. The reading of the results is performed daily. At the end of the experiment, the number and size of tumors formed after treatment are measured.

## Statistical analysis

The significant effect of agrocin on growth inhibition of *A. tumefaciens* was evaluated by Analysis of variance (ANOVA1) (factor: treatment), performed with the SPSS20 statistical software (IBM Corporation, Somers, NY, USA). The *Arcsin* of the inhibition percentage was used for statistical analysis and was calculated using the formula  $\text{Arcsin} = \sqrt{(\%I/100)}$ , where %I is the rate of bacterial growth inhibition.

## Results

### Morphology characterization

In the surveyed almond farm in the northwestern of Morocco, crown gall was observed in recently planted almond trees sourced from local nurseries. The incidence of the disease varied across different stations. Indeed, the rate of infestation in some sites exceeds 50% while in other sites it is between 10 to 40%. The most affected almond cultivars include the local variety Beldi, Ferragnes, and notably Marcona, which is the most sensitive of them. The Collected tumors are observed on the crown and root system of the tree. They are spherical, spongy and brown with a lignified surface (old tumor) and sometimes whitish (young tumor) and whose diameter varies from 1 to 10 cm (Figure 2).

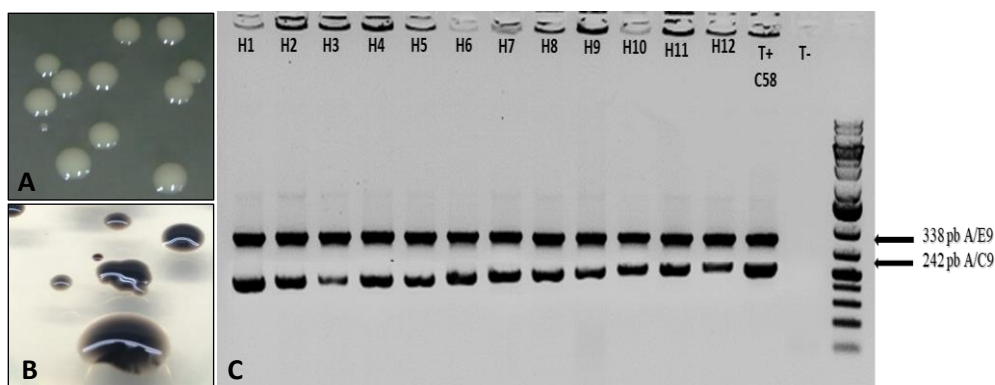


**Figure 2.** Symptoms of the crown gall of the almond trees: tumors developed on the trunk of Moroccan vines (INRA-Meknes).

Bacterial isolation from tumors and soil samples collected from the different locations allowed us to isolate out a total of 65 bacterial isolates on MG medium, which only 20 are preserved after the specific purification on MG+T. Colonies from MG medium alone (without tellurite) are creamy-white, convex, smooth, shiny and circular with entire mucoid edges (Figure 3A). On the MG+tellurite medium, the colonies are black with a white outline, bulging and sometimes mucous (Figure 3B).

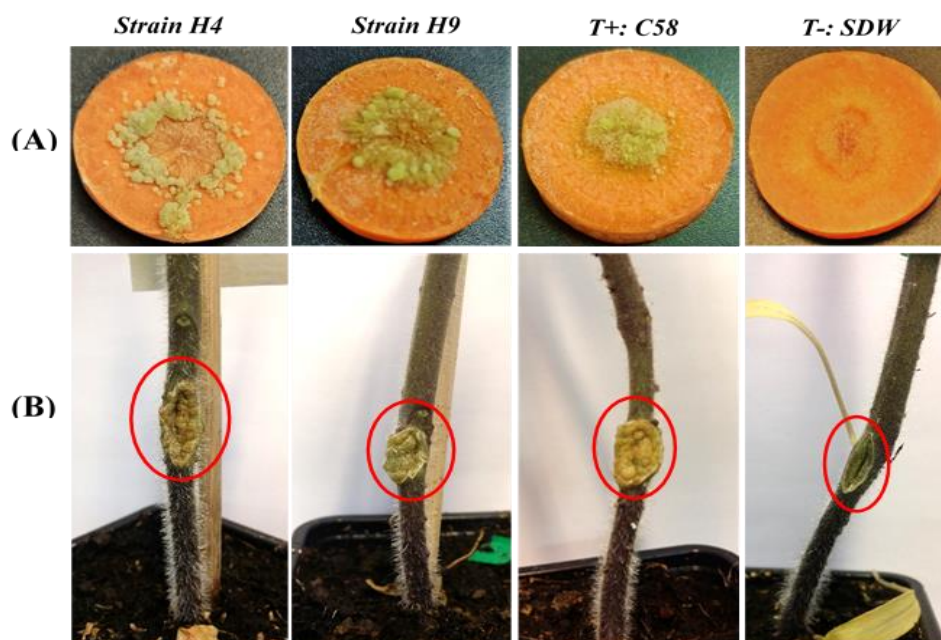
### **Molecular identification and pathogenicity identification**

Molecular characterization of isolates from tumors and soil was carried out *via* multiplex PCR using 2 specific primers A/C9 (242 bp) & A/E9 (338 bp) specific to the *virD2* genes located in the Ti plasmid. The Figure 4 showed the PCR profile of the amplification. The results show that the amplification of the *virD2* fragment of the *vir* region in the plasmid Ti (414 bp) is obtained for 12 strains isolated from tumours. However, the 8 isolates from the soil sample do not amplify with the two primers.



**Figure 3.** Morphological and molecular characterization of *Agrobacterium tumefaciens* isolated from tumors and soils samples. (A): colonies morphology on MG agar medium, (B): colonies morphology on MG+Tellurite agar medium, and (C): electrophoresis profil showing DNA amplification of the different isolates using A/E9 and A/C9 primers (Lane 1 and 12), a positive control representing by reference stain C58 (Lane 13), negative control (PCR mix without DNA).

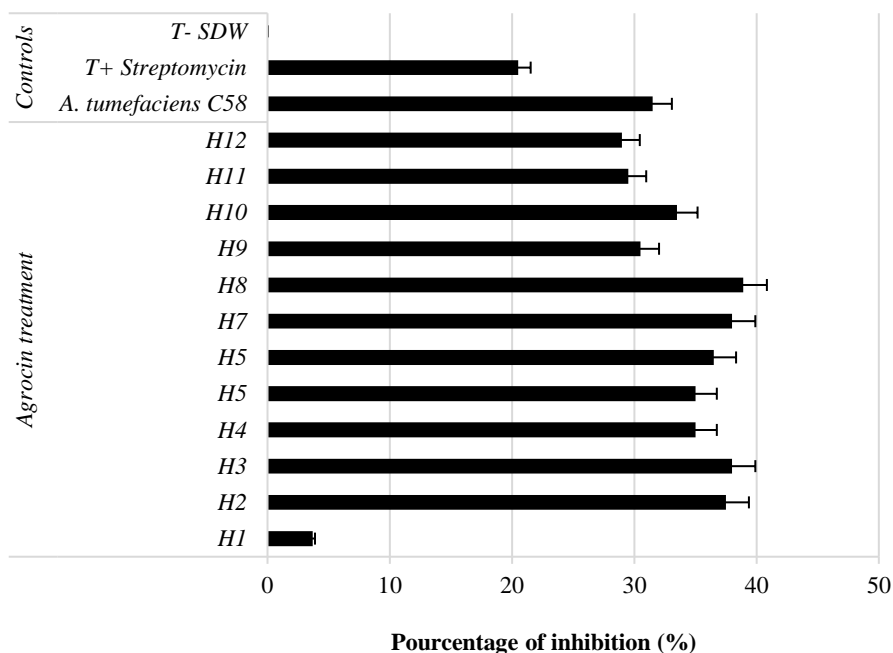
The results of the molecular characterization are compatible with those of the pathogenicity tests in tomato plants and carrot slices (Figure 4). All these isolates from tumours are pathogenic and induced typical tumours *in vitro* on tomato (Figure 4A) and carrots slices (Figure 4B). the isolates from soil sample are not able to produce tumours in the inoculated tomato plants and carrot slices.



**Figure 4.** Pathogenicity test *in vitro* on carrot slices (A) and tomato plants (B). Strains H4 and H9: *A. tumefaciens* isolated from tumors of almond tree, T+ (C58): positive control representing by the reference strain *A. tumefaciens* C58, and T- (SDW): negative control representing by the inoculation of the sterile distilled water.

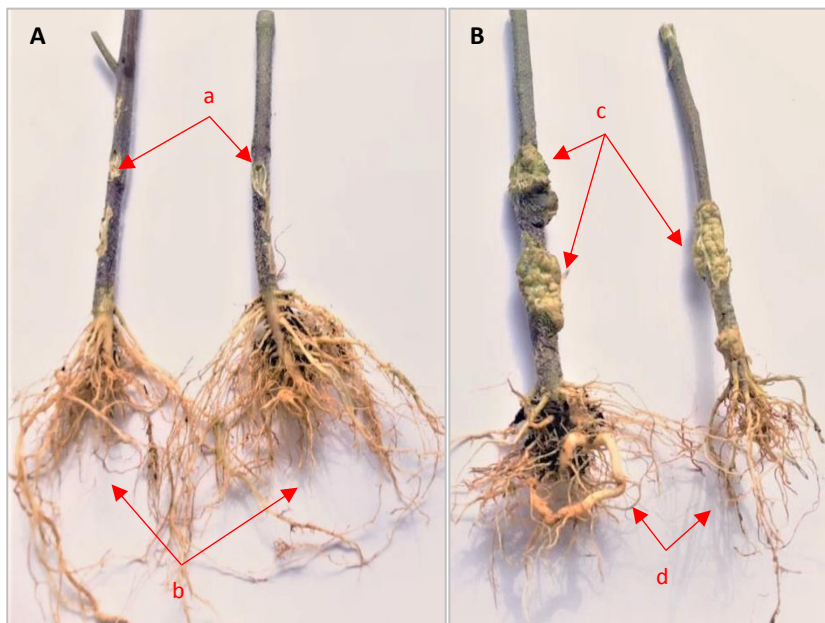
### Biocontrol activities

The confrontation test in YPG liquid medium between the bacterial antagonist *A. radiobacter* (strain K1026) and the reference strain *A. tumefaciens* (strain C58) showed a considerable inhibitory activity *in vitro*. The application of the agrocin extract from the confrontation test in the aromatogram test showed a high antagonistic activity toward the *A. tumefaciens* isolated from almond tree with a percentage of growth inhibition ranged from 29.5 to 38.9% compared with negative control (SDW 32mg/L: 0%) and positive control (streptomycin: 20.5%) (Figure 5).



**Figure 5.** Aromatogram test of the antibacterial activity of agrocin treatment against *A. tumefaciens* strains isolated from almond tree.

The inoculation of wounded sites of tomato plants using the strain K1026 of *A. radiobacter* as a prevent treatment, provide significant reduction in incidence and size of galls formed in response to subsequent inoculation with different strains of *A. tumefaciens* isolated from almond tree tumours comparatively with 100% gall incidence resulted with positive control (inoculation with *A. tumefaciens* C58 alone) (Figure 6). The Inoculation of tomatoes with strain K1026 not only prevented the development of tumors but also promoted the development of the root system and taproots (Figure 6).



**Figure 6.** Effect of the strain K1026 of *A. radiobacter* on incidence and size of galls induced by *A. tumefaciens* in tomato plant. (A): preventive treatment with the strain K1026, (B): positive control representing by the inoculation with *A. tumefaciens*. (a & b): the non-development of tumors in the inoculation site after treatment with the K1026 strain; (b): development of the root system and taproots. (c): development of tumours in the inoculated plant. (d): normal root system.

## Discussion

During the last decade, almond tree crown gall has become increasingly prevalent in all regions known for almond production in Morocco, causing serious damage and general discomfort among farmers. Surveys conducted at the stations visited during this study suggest that the bacterium, *A. tumefaciens*, which resides within the vascular tissue during its latency phase, has the potential to spread through plant material. This systemic presence becomes apparent when conditions prompt the onset of symptoms, signifying the infectious process (Burr et al., 1998). Consequently, the inherent systemic nature of *A. tumefaciens* complicates indexing and certification programs, inadvertently promoting the pathogen's dissemination through reproductive material (Habbadi et al., 2019). Nonetheless, the occurrence of the disease varies among the surveyed orchards, and this variability can be attributed to various factors including the host plant, the bacterium, soil composition, among others.

Among the most susceptible almond varieties in Morocco, Marcona, Beldi, and Ferragnes stand out, likely due to their high susceptibility. Generally, the resistance of the host plant depends partly on the cultivars and also on the degree of virulence of the pathogen. This is due to genetic factors related to the bacteria and the plant. According to Sparrow et al. (2004), the variability in resistance is primarily attributed to genetic factors, accounting for 95% of the observed variance, while environmental factors contribute to the remaining 5%. Furthermore, the responsiveness of host plants to tumorigenic agents displays diversity depending on their geographical source

(Pionnat et al., 1999). This phenomenon was elucidated in the study by (Benjama et al., 2002), wherein it was demonstrated that the apricot crop (the host plant of *A. tumefaciens*) exhibits considerable resistance in Moroccan conditions, both in soil deliberately inoculated with the pathogen and in naturally infested soil. Conversely, the same crop shows heightened sensitivity in countries like Tunisia (Trigui, 1984) and Turkey (Aysan et al., 2003). This difference in resistance can be attributed to variations in pedoclimatic and physico-chemical factors among different countries. Indeed, some soils are presumed to be conducive to crown gall while others are shown to be suppressive (Krimi et al., 2002).

Bacterial isolation from tumor and soil samples collected from diseased trees involves the application of various established techniques that rely on the utilization of selective and semi-selective culture media (Benjama et al. 2002). In our study, all isolates from either tumors or soil are purified on MG and MG+T medium; the latter promotes the growth of the targeted bacteria and facilitates their selection from a complex environment. The black appearance of colonies on MG+T medium is due to the incorporation of tellurite into the bacterial cell (Campillo et al., 2012).

In the present study, the characterization of the isolates from tumors and soil was initially performed by molecular analysis using two specific primers (A/C9 & A/E9) to the *virD2* region of the pTi (Haas et al., 1995). The results obtained demonstrated that all isolates from the tumors amplified with the two primers, which confirming the presence of the oncogenic element pTi and consequently indicating the pathogenicity of the *A. tumefaciens* isolates. The molecular test results were confirmed by the pathogenicity test in tomato plant and carrot slices where only *A. tumefaciens* demonstrated the ability to induce tumors on indicator plants. The correlation between the two tests (molecular and pathogenicity) for the identification of pathogenic isolates was demonstrated in the study work of by (Benjama, 2005) where he showed that strains of *A. tumefaciens* isolated from diseased almond tree and which present pTi are able to induce tumors on tomato and Kalanchöe plants. However, in other studies, the molecular tests for the detection of pTi and the pathogenicity test have been found to be incompatible; this was explained by the high host plant specificity in some strains of *A. tumefaciens* which present pTi whereas they are not able to produce tumors on tomato or tobacco plants (Anderson and Moore, 1979). It's worth mentioning that the choice of the indicator plant is a crucial factor in the pathogenicity test, considering factors such as the plant's growth time, the method of inoculation, the pathogen-host specificity, and the timing of symptom onset (Benjama, 2005).

Recently, the biological control of grapevine crown gall caused by *A. tumefaciens* has been the subject of several studies in view of the absence of other effective means to control this pathogen. The use of microorganisms in disease prevention has become a very interesting alternative method in disease control. Several bacterial species have been evaluated to have high *in vitro* antibacterial activity against *A. tumefaciens* (Bazzi et al. 1999; Farrand and Wang, 1992; Jones & Kerr, 1989) which can act directly (antibiosis and/or competition) or indirectly (induction of host plant resistance).

In our study, biological control utilizing *A. radiobacter* strain K1026 has demonstrated significant efficacy both *in vitro* and *in planta* against the tumorigenic agent *A. tumefaciens*. This bacterium effectively inhibits the growth of *A. tumefaciens* through the secretion of agrocin with a very high percentage of inhibition. Our results also

showed great efficacy *in planta* by reducing and/or eliminating the formation of tumors in the inoculation sites in indicator plants.

The use of *A. radiobacter* K84 was the first biological control model for crown gall caused by *A. tumefaciens* (Smith and Townsend, 1907). This biological control agent employs an antibiosis mechanism by producing an antibiotic which is agrocin 84 thanks to the presence of a specific plasmid to this species (pAg K84). This antibiotic acts by penetrating the target cells thanks to a permease which allows the two opines: nopaline and agropine to cross the membrane and enter the pathogenic bacterium (Kerr and Htay, 1974). Another strain within the *A. radiobacter* family, namely, K1026, was developed from the biological agent of control *A. radiobacter* K84 resulting in the production of a biopesticide which is the NoGall. This strain K1026 which was genetically modified from strain K84, was designed to avoid the transfer of plasmid pAgK84 to pathogenic *A. radiobacter* strains, significantly reducing the impact of K84, thereby ensuring the quality of biological control. This K1026 has been effectively employed in Australia (almond) and Spain (GF677 and cherry), Italy and France, on various stone rootstocks (López et al., 1989).

Another biopesticide was formulated using a pure culture of *A. radiobacter* mixed with peat. Its application on young root systems helps prevent the formation of crown gall; this product is very economical and offers the possibility of reducing the losses of the nursery sector which were 10 to 80% in sensitive plant species; this naturally occurring organism meets the criteria for a microbial control agent set out in the Registration Guidelines; experimental trials have demonstrated its capacity to reduce crown gall infections in nursery stock to as low as 1 or 2%; this reduction has a positive impact on the income of producers and the sector (Van Delden, 1989).

Biological control via the use of bacterial antagonists results from different types of interactions between microorganisms. Published information on the mechanisms of action of most biocontrol agents still remains incomplete due to the challenges in analyzing the interactions among the host plant, the pathogen, the antagonist and other microorganisms (Spadaro and Gullino, 2004). To date, several mechanisms of action have been described, including: antibiosis, competition for space and nutrients, induction of defense mechanisms in the host plant, quorum quenching and parasitism. However, the majority of antagonists do not rely solely on a single mechanism; disease control often results from a combination of several mechanisms (Siddiqui and Mahmoud, 1999; Gonzalez, 2007).

## Conclusion

The importance of this research work is underscored by the importance of the almond tree in Morocco, where it holds a prominent position in the national agricultural production. On the social level, almonds contribute significantly to farmers' income and provide employment opportunities, both in the fields and in marketing and promotion channels. However, crown gall of almond tree still challenging disease in almond production worldwide due to the absence of an effective means of control. In the present study, we investigate the efficacy of the *A. radiobacter* strain K1026 isolated from the NoGall and the antibiotic agrocin against Moroccan isolates of *A. tumefaciens* from infected almond tree. The results of the *in vitro* and *in planta* experiments showed that potent antagonist can be a good potential sources of bioactive metabolite “agrocin” and an important alternative to control almond tree crown gall disease. They provide a crop protection with a low environment risk associated.

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