Biological Control of Grapevine Crown Gall Caused by *Allorhizobiumvitis* using Bacterial Antagonists

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Abstract: ***The potential use of bacterial antagonists isolated from different area in Morocco for the biological control of grapevine crown gall caused by Allorhizobiumvitis was investigated. In vitro analyzing the activity of 90 bacterial isolates towards Allorhizobiumvitis strain S4 resulted in a selection of 26 biocontrol agents. The isolated were tested for their ability in vitro to inhibit the growth of the pathogen. Among these isolates 12 antagonists are efficient. Molecular identification of selected isolates, using rDNA 16S sequencing, show that the antagonists belong to different genera as Bacillus spp., Pantoea sp., Rahnella sp., Acinetobacter spp. and Enterobacter sp. Four antagonists were tested for their antagonistic effect in planta; they exhibited considerable inhibitory activity to reduce the incidence of galls in tomato and squash fruits. Rahnellaaquatilis and Pantoeaagglomerrans reduced the incidence of crown gall up to 100% both in tomato and squash fruits. Bacillus subtilis reduced the incidence of gall development to 75% in squash fruits and 60% in tomato. Whereas, Acinetobacter calcoaceticus reduced incidence of gall formation to 65% in squash fruits and 47% in tomato. Consequently, the treatment with bacterial antagonists may be used as an effectiveness alternative to control crown gall disease.***

Keywords: Crown gall, grapevine, *Allorhizobiumvitis*, biological control, bacterial antagonists, Morocco

1. Introduction

Crown gall of grapevine is a bacterial disease caused by *Agrobacteriumvitis* [39], recently renamed *Allorhizobiumvitis* [37]- [38]. This disease presents a serious problem to grapevine production in several regions of the word [8]. *A. vitis* can survives systemically in the plant tissues until conditions become suitable for gall development [6] and, therefore, is disseminated in propagating plant material [30]- [50]. The tumorigenicity of *A. vitis*is determined by the presence of a set of oncogenes called the T-DNA that are carried on a large tumor-inducing (Ti) plasmid [51]. The T-DNA is transferred and integrated into the plant cell genome; the genes encode enzymes that stimulate production of plant hormones (auxin and cytokinin) that cause the proliferation of plant cells and development of tumors. The T-DNA also contains genes responsible for the biosynthesis of opines, specific carbon and nitrogen sources for *A. vitis* development [1]- [31].

The systemic survival of *A. vitis* in grapevine makes it difficult to controlthe disease. The older vines usually survive the infection, although they show stress symptoms, young vines that develop a tumor at their graft union often die [48]. The damage could threaten the harvest and grape quality in the absence of appropriate crop management [11]. Until now, the control of crown gall of grapevine is based on viticultural criteria as well as on the indexing and certification of propagation material [3]- [49]. Currently, there are no real effective chemical treatments to control the crown gall of grapevine. Generally, chemical options of this disease are limited to the use of disinfectants (sodium hypochlorite) copper or antibiotics [3]- [24]- [35]. However, these treatments can kill the bacteria on gall surfaces, and fail to control the systemic survivalof *A. vitis* in the vascular tissue of the grapevine [7]- [10].

Recently, the biological control of grapevine crown gall has been the subject of several studies due to the absence of other effective means of control. The use of microorganisms to prevent the disease, offers an attractive alternative for the management of crown gall disease. Numerous bacterial antagonists with biological control activity have been evaluated against *A. vitis* [12]- [13]- [15]- [22]- [28]- [43], which can have direct (antibiosis, competition for target sites or nutrients) or indirect action (induced resistance in the host). Some of these antagonists are epiphytic; they colonize the rhizosphere and the root of host, while others cans also survive systematically in the hot and becomes endophytes [14]- [34]- [46]. The use of *Agrobacterium radiobacter* strain K-84 as a biological treatment was the first model used successfully against crown gall on several plant species caused by *Agrobacterium tumefaciens* [45]; however, this antagonist is not effective for preventing infections on grape caused by *A. vitis* [6]- [25]. Recent studies have demonstrated that the use of nonpathogenic strains of *A. vitis* inhibit growth of most tumorigenic strains of *A. vitisin vitro* and can also inhibit crown gall on grapevine in the greenhouse [21]- [32]- [47]- [53]. The main purpose of this study was to evaluate the practical potential of biological control of some bacterial antagonists isolated from Morocco against *A. vitis* strain S4 using *in vitro* and *in planta.*

2. Material and Methods

Bacterial strain and culture conditions

The bacterial strains used in this study are listed in Table 1. *A. vitis* strain S4 used in this study was cultivated on

MG medium [36](D-mannitol, 5g/L; L-glutamic acid, 2g/L; KH_2PO_4, 0.5g/L; NaCl, 0.2g/L; $MgSO_4 \times 7H_2O$, 0.2g/L; Yeast extract, 0.5g/L; Agar, 15g/L; pH=7) and incubated, for 24 hours, at 28°C.

Antagonistic bacteria were selected among a collection of 90 isolates, which were isolated from different plants in different region in Morocco. They belong to the collection of laboratory of Phytobacteriology and Biological Control of the National Institute of Agronomic Research of Meknes. The selection of bacterial antagonist was based on the ability to inhibit growth of *A. vitis* in YPGA medium(yeast extract, 5g/L; peptone, 5g/L; glucose, 10g/L; agar, 15g/L). The strains were cultured at 28°C on YPGA and incubated, for 24 hours, at 28°C.

Antagonistic activity *in vitro*

A 100 µl sample of the *A. vitis* strain S4 suspension (10^7 CFU/mL) was inoculated, using the flooding method, on YPGA medium. Sterile filter paper discs (5mm diameter) was impregnated by bacterial cream of antagonist and placed in Petri dishes, either directly onto the center of the culture medium. The plates were incubated in the upright position at 28°C for 24 hours. The filter soaked with 2µL of sterile distilled water was served as negative control and filter impregnate with streptomycin (32mg/L) was served as positive control. After incubation, the inhibition zone around each disc was measured and the percentage inhibition was calculated using the following formula [41]:

$$\textbf{Percent inhibition}\ (\%) = \left[\frac{(\textbf{Rate without inhibitor} - \textbf{Race with inhibitor})}{\textbf{Rate whitout inhibitor}}\right] \times \mathbf{100}$$

Molecular identification of antagonistic bacteria

The identification of bacterial antagonists was made for 12 antagonistic bacteria, presenting a height antibacterial activity against *A. vitis* strain S4, using conserved 16S rRNA gene for the detection and identification of bacteria. The DNA extraction was made using alkaline method [42]. Pure culture genomic DNAs were extracted from bacteria grown overnight at 28°C in YPGA. One colony of each bacteria isolated was mixed with 10µl of (20 mMNaOH) and incubated at 37°C for 5 minutes. The bacterialyses cells were stored at 4°C until they use.

Amplification was carried with primers F809pA (AGAGTTTGATCCTGGCTCAG) and F810pH (AAGGAGGTGATCCAGCCGCA). Standard PCR was carried out in a 60µl reaction volume containing 38.6µL H_2O, 6µl (2mM) DNTPs, 1.2µl (2mM) $MgCl_2$, 3µl DMSO (Dimethyl sulfoxide), 1µl (10 µM) of each primer, 0.2µl Taq DNA polymerase (Invitrogen, France) and 3µl of lyses cells. The PCR was performed using the following program: initial denaturation at 94°C for 5min, followed by 35 cycles of denaturation at 94°C for 1min, annealing at 55°C for 1min and extension at 72°C for 1min, followed by an additional extension at 72°C for 5min.

Electrophoresis was performed in 1% agarose gel. The gel was stained with ethidium bromide. Fragments were visualized with an ultraviolet (UV) transilluminator, and the gel was photographed. The 16S gene of each isolates was sequenced (GenoScreen Lille-France) and analyzed using NCBI-BLAST software [2].

Biocontrol activity *in planta*

Four isolates that yielded the greatest percentage of inhibition for growth of *A. vitis* stain S4 were selected to demonstrate its biocontrol activity *in planta*as a preventive treatment against tumor development. These isolates were examined for their ability to suppress gall formation by *A. vitis* in tomato (*Solanum lycopersicum* L.) and summer squash fruits (Cucurbita pepo cv. Eskandarany). Each test plant was inoculated with one of the biocontrol agents.

In the case of tomato, the inoculation was made by 10 µl of specific antagonistic suspension (10^7CFU/mL) of 24 hours bacterial culture in stem internodes of tomato 2-3 weeks after transplanting. After the liquid was absorbed by the plant tissue, the wounded sites were wrapped in Parafilms. After 24 hours, each site of inoculation was rewounded and 10 µl of *A. vitis* strain S4 suspension was introduced in each site. Non-treated plants inoculated with *A. vitis* S4 or with sterile distilled water were used as a positive control and negative control, respectively. After the suspension was absorbed into the wound, the stem was again wrapped in Parafilms. The inoculated plants were maintained in greenhouse at 27°C during 3-4 weeks. Number and size of formed galls were recorded.

In the case of squash fruits, uniform fruits (10 to 15 cm of length and 3 to 5 cm of width) were stabbed by toothpick to make holes at five sites distributed over two rows (5 sites/row) per fruit. The inoculation was performed in the same manner as in tomato leaves. The treated fruits were maintained in plastic containers with transparent plastic covers and kept at 28°C. The presence or absence of tumors was visible 7 weeks or 10 days after inoculation and the number and size of formed galls were recorded.

Statistical Analysis

The significant effect of bacterial antagonists on growth inhibition of *A. vitis* was evaluated by Analysis of variance (ANOVA1) (factor: treatment), performed with the SPSS 20 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.

3. Results

Antagonistic activity *in vitro*

Among 90 isolates, many isolates showed antagonistic activity toward *A. vitis* strain S4 in variable degree. Among these isolates, only 26 isolates exhibited considerable inhibitory activity (Figure 1). The mean values of percent of inhibition resulted from these isolated fluctuated between 13.3 and 39.8%. The greatest inhibition resulted from isolate 2515-3 (39.8%) flowed by isolate

2332-A1 (32.2%) (Figure 2). The isolates 2626-5, 2510-8, 2627-1, 2510-9, 2027-1, 2546-4, 2066-7, 2328-B5, 2021-12 and 2021-2 showed moderately antagonistic reaction indicated by the percent of inhibition values (25.5, 16.6, 27.7, 24.6, 27.7, 27.7, 27.77, 27.9, 22.22, 20% respectively).

Molecular identification of antagonist

Further molecular analysis was carried out using universal primers F809PA and F810PH targeted gene 16SrDNA of 1477 bp (Figure 3). Analysis of the 16SrDNA sequence, by BLAST-NCBI, of 12 bacterial antagonist perentinga high efficacy against *A. vitis* S4 and originating from different samples and locations in Moroccoare shown in Table 2. According to the sequencing results of 16SrDNA, the bacterial antagonists belongs to different species from different genus: *Bacillus*spp., *Pantoea*spp., *Rahnella*spp., *Acinetobacter*spp. and *Enterobacter*spp. The antagonists strain belong to *Pantoea* and *Rahnella* genus possessed a 16S rDNA sequence with ≥ 99% similarity to that of genus members. The *Enterobacter* and *Acinetobacter* antagonists exhibited ≥ 97% 16S rDNA similarity whit this genus.

Biocontrol activity *in planta*

Inoculation of wounded sites on tomato and squash fruits with antagonistic bacterial isolates, using as a prevent treatment, provide significant reduction in incidence and size of galls formed in response to subsequent inoculation with *A. vitis* S4 comparatively with 100% gall incidence resulted with positive control (inoculation with *A. vitis* S4 alone). There was significant difference between the tested antagonists in the prevention of galls formation on the different tested plants (Figure 4, Figure 5). The antagonists 2332-A1 (*Rahnellaaquatilis*) and 2066-7 (*Pantoeaagglomerans*) reduced the incidence of crown gall up to 100% in tomato and squash fruits. The isolate 2515-3 (*Bacillus subtilis*) reduced incidence of crown gall to 75% in squash fruits and 60% in tomato. Isolate 2328-B5 (*Acinetobacter calcoaceticus*) reduced incidence of gall formation to 65% in squash fruits and 47% in tomato.

4. Discussion

In this study, the efficacy of treatment with bacterial antagonists was demonstrated by the reduction *in vitro* as well as *in planta*. From 90 tested microbial antagonists, only 26 isolates exhibited an antibacterial activity *in vitro* against *A. vitis* S4. The idea provided to select effective biocontrol strain, among the collection, capable to control *A. vitisin vitro* and *in planta*. Numerous studies were conducted to evaluate the antibacterial activity of some microbial antagonists and their potential for use in biocontrol programs for the management of grapevine crown gall [52]. The nonpathogenic strains of *A. vitis* were the first model has been studied as a biological control agent of grapevine crown gall. Numerous strains of *A. vitis* were effective *in vitro* and *in vivo* against tumorigenic strains. *A. vitis* strain F2/5 was the most effective strain capable to inhibit the growth of pathogenic strains *in vitro* and galls development in grapevine [4]- [9]- [10]- [26]. The mechanism of action of *A. vitis* strain F2/5 is the competition for attachment sites on grape and antibiosis mechanisms by producingantibiotic (agrocin) [9].

Among the screened isolates in this study, 12 antagonistic bacteria, with strong antibacterial activity *in vitro* against *A. vitis* S4, were selected and identified in the genera *Bacillus* (*B. subtilis* and *B. cereus*), *Pantoea* (*P. agglomerans*), *Rahnella* (*R. aquatilis*), *Acinetobacter* (*A. calcoaceticus*and *A. venetianus*) and *Enterobacter* (*E.ludwiqii*). Moreover, the result of the present study demonstrates that the bacterial antagonists tested can inhibit the growth of *A. vitisin vitro*. However, *B. subtilis* (2515-3) is the most effective biocontrol agent *in vitro*followed by *R. aquatilis* (2332-A1) and *P. agglomerans* (2066-7). The efficacy of these species has been documented for antibacterial activities *in vitro* against many pathogens include *A. vitis*. In the study work of Sholberg*et al.* [43], they demonstrate that two species of *Bacillus* spp. (EN63-1 and E71-1) can inhibit bacterial growth of *A. vitis in vitro*. The efficacy of *R. aquatilis* was also evaluated against *A. vitis* by Bell *et al.* [5];Chen *et al.* [12]- [13]; when they showed that this antagonist can exhibit a high antibacterial activity against *A. vitis* strains *in vitro* and *in planta*. In the research work of Kenneth *et al.* [28], they show that the *P. agglomerans* inhibit the growth of *A. vitisin vitro* and can inhibit galls development *in planta*.

Some bacterial antagonists are known to have antibacterial activities *in vitro* but the biocontrol effectiveness may not be expressed under *in planta* conditions [23]. In the present research, the selected antagonistic isolates proved to be efficient *in vitro* and under *in planta* conditions in variable degree. *R. aquatilis* (2332-A1) and *P. agglomerans* (2066-7) are the most effective antagonists capable to exhibit considerable inhibitory activity toward gall formation both in tomato and squash fruits (100% of reduction). This correlation between *in vitro* and *in planta* results have been documented in the study of Bell *et al.* [5], Chen *et al.* [12]- [13], Tolba and Solimane [52] and Gupta *et al.* [20]. Interestingly, the antagonistic isolate *B. subtilis* (2515-3), which present the most effective antagonist *in vitro*, reduce the galls development only to 60% in tomato and 75% in squash fruits. This lack of correlation between *in vitro* and *in planta* tests, in the control of *A. vitis*, has been documented in other studies with other antagonists [4]- [29]- [52]. For the isolate *A.calcoaceticus* (2328-B5) reduced the incidence of crown gall to 65% in squash fruits and 47% in tomato. This experiment indicated that the different in the performance of antagonistic bacteria has been attributed to variability in the physical and chemical properties within the niches occupied by biocontrol agents and by the host, which affect the colonization and efficacy of biocontrol agent [40].

Biological control using antagonistic bacteria result from many different types of interactions between organisms. The information about the mechanism of action for most of the antagonists is still incomplete due to difficulties in analyzing the complex interactions between host, pathogen, antagonist and other microorganisms present [46]. Several biocontrol mechanisms have been described including antibiosis, competition for nutrients and space, induction of mechanisms of resistance in the host plant, quorum quenching and direct interaction between the antagonist and the pathogen including parasitism. Actually, most biocontrol agents not only use one mechanism of biocontrol, but disease control is the result of a combination of several mechanisms [18]- [44]. Bacterial antagonists members of the genus *Bacillus* were reported, in many studies, to exhibit an antagonistic activity by producing a wide range of secondary metabolites such as antibiotics (iturin and gramicidin) [16]- [19], non-volatile and volatile compounds and lytic enzymes [17]- [43]. *R. aquatilis* was reported to synthesis a secondary compounds (antibiotic) with wide range of action against bacteria and fungus [33]. *P. agglomerans* have a preventive effect by competition mechanism to acquire nutrients from the environment than the pathogen; is the most antagonist presenting a higher competitive ability used against many pathogenic bacteria and fungus [27].

5. Conclusion

In conclusion, results obtained in the present work show that the bacterial isolates identified may be considered as potential sources of bioactive metabolites and an important alternative to control grapevine crown gall disease. They provide a crop protection with a low environment risk associated. Future studies are recommended to develop a mass protection method of the bacterial antagonists, to find the appropriate formulation that allow to increase biocontrol activity and ensure its stability and to develop a bacterial pesticides used in the control of crown gall of grapevine.

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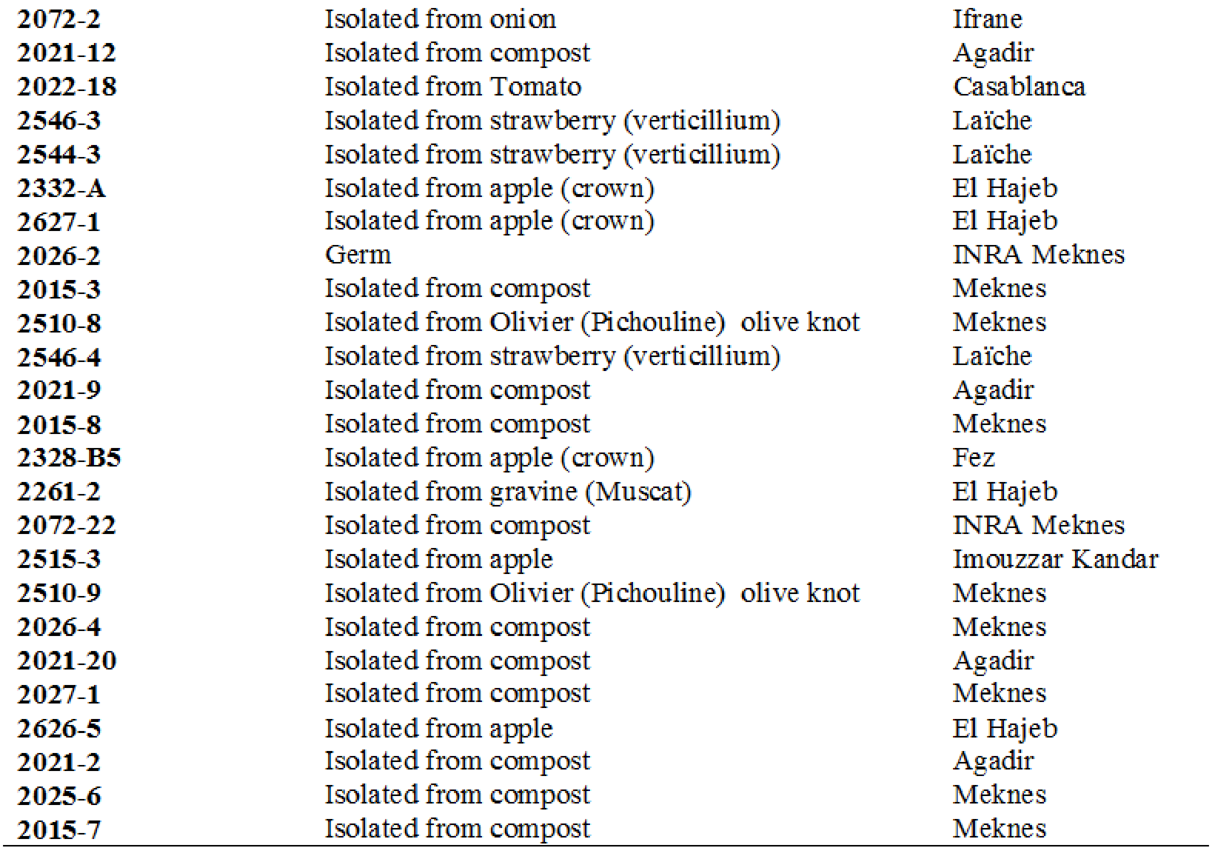
Table 1: strains used in this study



Strains	Relevant characteristics	Sampling location
***Allorhizobium vitis* S4**	Isolated from black raspberry	Hungary
2066-7	Isolated from Olivier (Pichouline) olive knot	Taounat
2072-2	Isolated from onion	Ifrane
2021-12	Isolated from compost	Agadir
2022-18	Isolated from Tomato	Casablanca
2546-3	Isolated from strawberry (verticillium)	Laïche
2544-3	Isolated from strawberry (verticillium)	Laïche
2332-A	Isolated from apple (crown)	El Hajeb
2627-1	Isolated from apple (crown)	El Hajeb
2026-2	Germ	INRA Meknes
2015-3	Isolated from compost	Meknes
2510-8	Isolated from Olivier (Pichouline) olive knot	Meknes
2546-4	Isolated from strawberry (verticillium)	Laïche
2021-9	Isolated from compost	Agadir
2015-8	Isolated from compost	Meknes
2328-B5	Isolated from apple (crown)	Fez
2261-2	Isolated from gravine (Muscat)	El Hajeb
2072-22	Isolated from compost	INRA Meknes
2515-3	Isolated from apple	Imouzzar Kandar
2510-9	Isolated from Olivier (Pichouline) olive knot	Meknes
2026-4	Isolated from compost	Meknes
2021-20	Isolated from compost	Agadir
2027-1	Isolated from compost	Meknes
2626-5	Isolated from apple	El Hajeb
2021-2	Isolated from compost	Agadir
2025-6	Isolated from compost	Meknes
2015-7	Isolated from compost	Meknes

Table 2: 16 rDNA results of 12 bacterial antagonists tested in this study

Code	Species	GenBank accession No
2515-3	*Bacillus subtilis*	KJ592619.2
2626-5	*Pantoea agglomerans*	KJ781904.1
2510-8	*Pantoea sp*	HQ396801.1
2332-A1	*Rahnella aquatilis*	KM241863.1
2627-1	*Acinetobacter calcoaceticus*	KP170504.1
2510-9	*Pantoea ananatis*	KM977993.1
2027-1	*Bacillus cereus*	KR493006.1
2546-4	*Enterobacter ludwiqii*	LC015543.1
2066-7	*Pantoea agglomerans*	KJ781904.1
2328-B5	*Acinetobacter calcoaceticus*	KP170504.1
2021-12	*Acinetobacter venetianus*	KP009554.1
2021-2	*Bacillus cereus*	KR493006.1

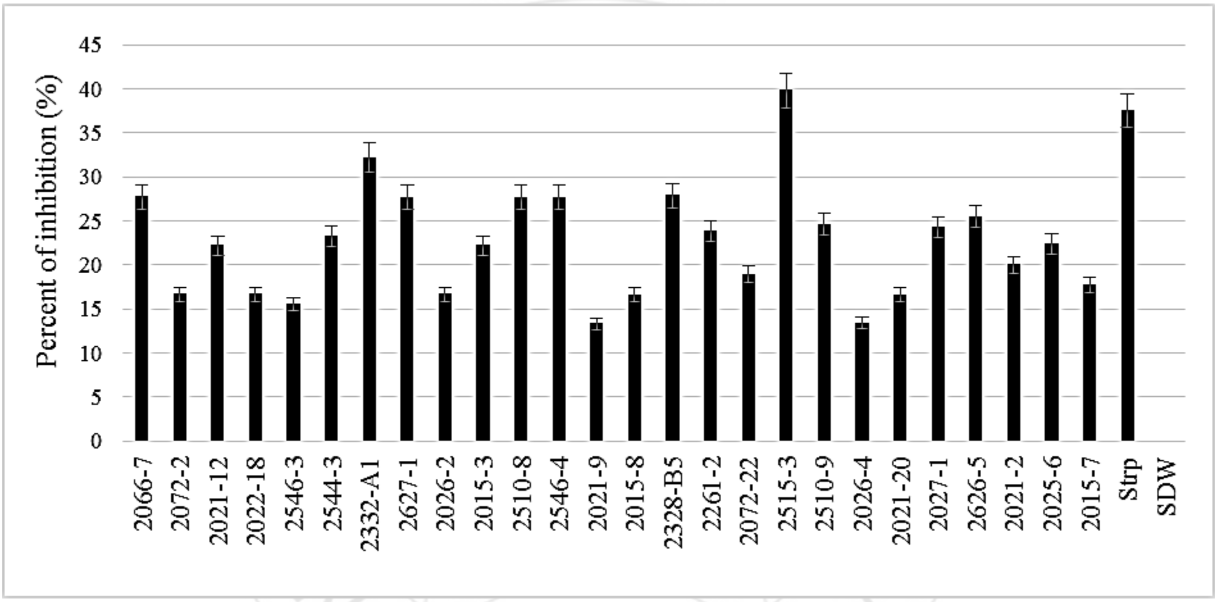


Figure 1: *In vitro* percent of inhibition caused by bacterial antagonists against *Allorhizobiumvitis* (strain S4). Strep: streptomycin antibiotic, SDW: sterile distilled water

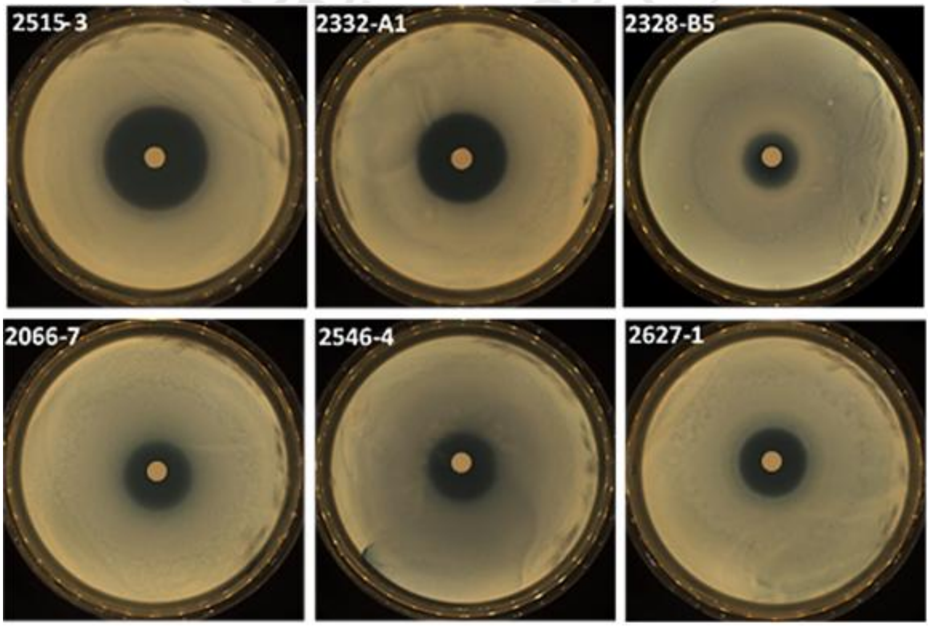


Figure 2: Inhibition zones resulted from challenge of bacterial isolates toward *Allorhizobiumvitis* (strain S4)

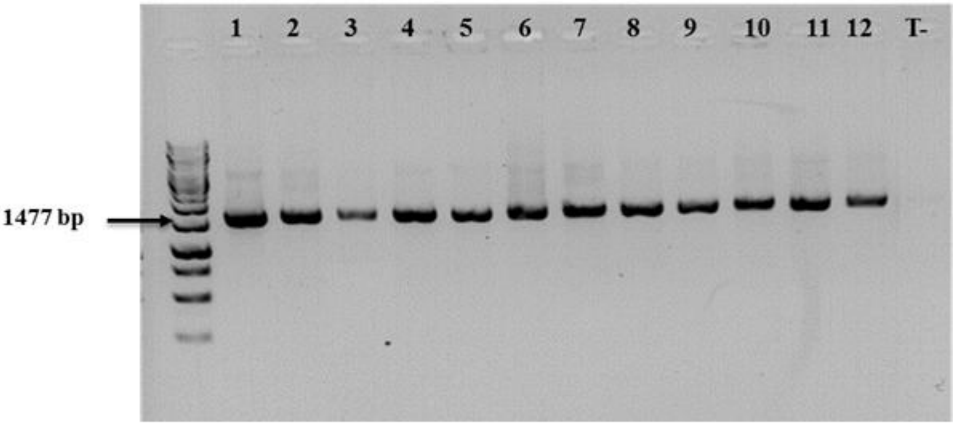


Figure 3: Electrophoritic profile of bacterial antagonits: (1) 2515-3. (2) 2626-5. (3) 2510-8. (4) 2332-A1. (5) 2627-1. (6) 2510-9. (7) *2027-1*. (8) 2546-4. (9) 2066-7. (10) 2328-B5. (11) 2021-12. (12) 2021-2. (T-) negative control

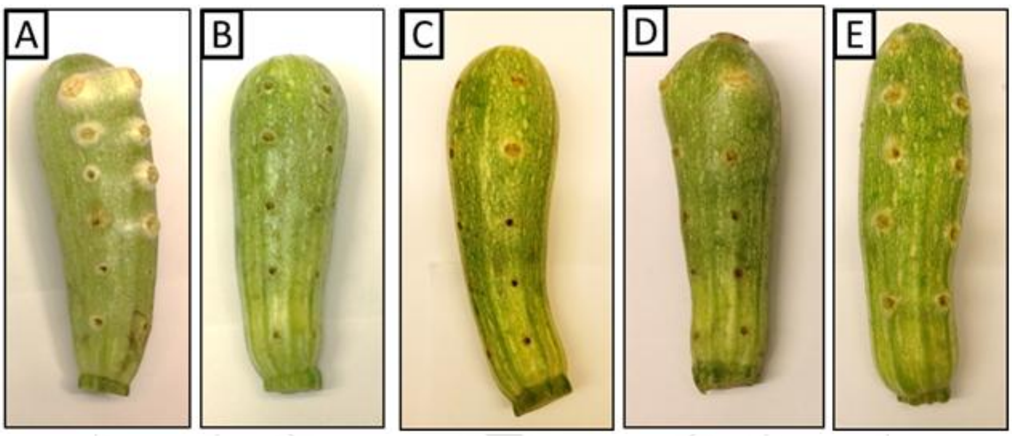


Figure 4: Effect of four antagonistic bacterial isolates on incidence and size of galls induced by *Allorhizobiumvitis*strain S4 in squash fruits. (A) positive control (inoculation with *A. vitis* S4), (B to E) preventive treatment with bacterial antagonists; (B) 2332-A1 (*R. aquatilis*), (C) 2066-7 (*P. agglomerans*), (D) 2515-3 (*B. subtilis*) and (E) 23228-B5 (*A. calcoaceticus*)

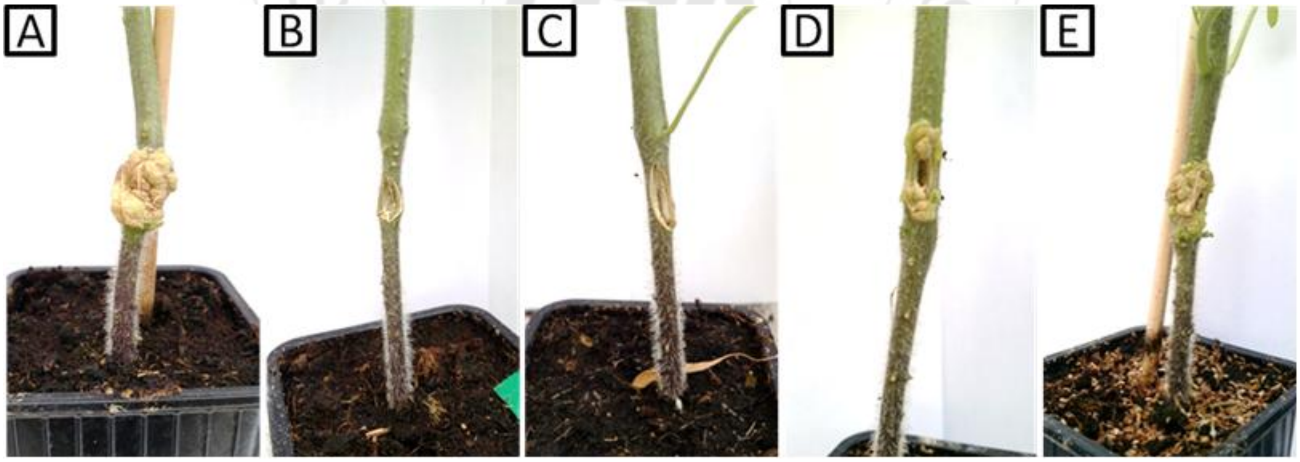


Figure 5: Effect of four antagonistic bacterial isolates on incidence and size of galls induced by *Allorhizobiumvitis*strain S4 in tomato plant. (A) positive control (inoculation with *A. vitis* S4), (B to E) preventive treatment with bacterial antagonists; (B) 2332-A1 (*R. aquatilis*), (C) 2066-7 (*P. agglomerans*), (D) 2515-3 (*B. subtilis*) and (E) 23228-B5 (*A. calcoaceticus*)