

Rapid and sensitive methods for detection of *Allorhizobium vitis*, causal agent of grapevine crown gall

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Abstract— A rapid method and sensitive methods for extraction of bacterial DNA from pure culture and directly from plant material were compared in polymerase chain reaction with specific primers VCF3/VCR3 to see the reliable method that can be used in the detection of tumorigenic strain of *Allorhizobium vitis* causal agent of grapevine crown gall. From the three tested methods of DNA extraction from pure culture, the alkaline method is the most effective technique for the extraction presenting a high sensitivity with a detection threshold equal to 5.10^4 CFU/ml. Five different protocols for extracting bacterial DNA from plant tissues of infected tomato, based on the use of an extraction buffer, were tested to see its usefulness in detecting pathogenic strain of *A. vitis* S4. Two protocols based on the use of Triton X-100 and Tween 20 were efficient for detecting *A. vitis* S4 directly from tomato tumors with a sensitivity of 10^3 CFU/ml for the both protocols. Consequently, these protocols were proposed as specific protocols for the detection of tumorigenic strain of *A. vitis* from symptomatic and asymptomatic plants.

Keywords— Crown gall, grapevine, *Allorhizobium vitis*, DNA extraction, detection, detection threshold.

I. INTRODUCTION

Crown gall of grapevine caused by *Allorhizobium vitis* (Moussavi et al., 2014, 2015), previously named *Agrobacterium vitis* (Ophel and Kerr 1990); is an economically important disease and one of the most serious bacterial diseases affecting grape production in several countries (Burr and Otten, 1999). The

tumorigenicity of *A. vitis* is an endogenous transfer into plant cells of the T-DNA (transferred DNA), part of a large tumor-inducing (Ti) plasmid (Thomashow et al. 1984). The T-DNA integrated into the plant cell genome, contains genes responsible for the biosynthesis of hormones (auxins and cytokines) leading to gall development and genes coded for biosynthesis of opines used as specific carbon and nitrogen sources for *A. vitis* development (Lacroix and Citovsky, 2013). The development of galls obstructs vascular tissue and restricts movement of water and nutrients into the vine above the gall, which affects grapevine growth and yield (Schroth et al., 1988).

A. vitis is adapted to survive in the plant tissues without causing tumors until conditions become favorable for gall development. Therefore, the disease spreads out by asymptomatic propagating materials (Kuzmanovic et al., 2014) which is necessary to develop a reliable detection method of the pathogen in both symptomatic and asymptomatic plant materials and in the soil to efficiently prevent the disease (Bini et al., 2008). Generally, the detection methods of *A. vitis* are based on their isolation on the semiselective culture media and pathogenicity tests but these methods can take many weeks for results (Johnson et al., 2013, Shams et al., 2012). Polymerase chain reaction (PCR) is the most reliable technique to improve sensitivity, specificity and rapidity for the detection of bacteria which targets genes that are found only in *A. vitis* by the use of specific primers (Johnson et al., 2013; Shams et al., 2012, 2013).

Several PCR protocols have been developed and successfully used for detection of tumorigenic strains of *A. vitis* directly from plant hosts with the use of primers, which target genes localized on Ti plasmid, *vir* or T-DNA regions (Bini et al., 2008). Generally, the methods of extraction of bacterial DNA from plant tissues are based to the use of a DNA purification Kit specific to *A. vitis* and characterized to inhibit the action of plant polyphenols that inhibit the PCR reaction (Bini et al., 2008). This method is very expensive and needs several steps for purification of the DNA; for this reason, many studies were conducted to develop other techniques of DNA extraction directly from infected plants (Llop et al., 1999; Szegedi and Bottka, 2002).

The main purpose of this study was to compare the extraction procedures of DNA from pure culture for efficient routine detection of *A. vitis* and to evaluate and optimize protocols of detection of *A. vitis* used Bio-PCR and PCR reaction directly for infected plant with the study of threshold of detection of the pathogen.

II. MATERIAL AND METHODS

Bacterial strain and culture conditions

The bacterial strain used in this study is *A. vitis* strain S4 (sequenced strain) isolated from black raspberry in Hungary (Popoff et al., 1984). *A. vitis* S4 was cultivated on MG medium (Moore et al., 2001) (D-mannitol, 5g/L; L-glutamic acid, 2g/L; KH_2PO_4 , 0.5g/L; NaCl, 0.2g/L; $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, 0.2g/L; Yeast extract, 0.5g/L; Agar, 15g/L; pH=7) and incubated, for 24 hours, at 28°C.

Pathogenicity and hypersensitivity tests

The pathogenicity of strains *A. vitis* S4 was studied by inoculating plants of tomato (*Solanum lycopersicum* L.). The inoculation was made by 10 µl of suspension (10^7 CFU/mL) of 24 hours bacterial culture in stem internodes of tomato 2-3 weeks after transplanting. Inoculated plants were maintained in greenhouse at 27°C during 3-4 weeks.

The hypersensitivity reaction was determined on tobacco (*Nicotiana tabacum* cv. Xanthi). 200 µl of bacterial suspensions (10^7 CFU/ml) were infiltrated on tobacco leaf by a needleless syringe. Sterile water was used as a negative control. The tobacco plants were kept in a growth greenhouse. Development of necrosis was scored over a period of 4 to 5 days.

DNA extraction from pure culture

Three extraction procedures were used to obtain DNA for Bio-PCR analysis:

The first protocol was based to use alkaline method (Shams et al., 2013). From bacteria grown overnight at 28°C in MG medium, one colony of *A. vitis* S4 was mixed with 10 µl of NaOH (20 mM) and incubated at 37°C for 5

minutes. The *A. vitis* lysed cells were stored at 4°C until they use.

The second protocol was based on the lysis of the bacterial cells by heating a bacterial suspension of 10^8 CFU/ml at 100 °C for 15 min. The lysed cells were stored at 4°C until they use (Hannou et al., 2013).

The third protocol is based on the lysis of the cells by thermal shock by heating the bacterial suspension for 15 min at 100 °C then cooling in ice for 5 min and centrifuging at 10000 g for 1 min. The resulting supernatant was used for PCR (Pastrick and Rainay, 1999; Ameur et al., 2014).

Bio PCR-pTi

Specific primers VCF3 (GGCGGGCGYGCYGAAGRAARACYT) and VCR3 (AAGAACGYGGNATGTTGCATCTYAC) were used to identify pathogenic strains of *A. vitis* by the detection of plasmids (pTi); they amplify a DNA fragment of 414bp of the *virC1* and *virC2* genes (Kawaguchi, 2009). Standard PCR was carried out in a 60 µl reaction volume containing 38.6 µl H_2O , 6 µl (2mM) dNTPs, 1.2 µl (2mM) MgCl_2 , 3 µl DMSO (Dimethyl sulfoxide), 1 µl (10 µM) of each primer, 0.2 µl Taq DNA polymerase (Invitrogen, France) and 3 µl of lysed cells from each extraction protocol were tested. In order to test the possibility to detect *A. vitis* without carrying extraction of the DNA, two other methods were tested; the first was to add a small colony directly in master mix using toothpick; and the second method was to add 3 µl of a bacterial suspension of (10^8 CFU/ml) into the mix. In all five protocols were tested to detect *A. vitis*.

The PCR was performed using the following program: initial denaturation at 95°C for 5min, followed by 35 cycles of denaturation at 95°C for 1min, annealing at 57°C for 1min and extension at 72°C for 1min, followed by an additional extension at 72°C for 3min. Electrophoresis was performed in 1.5 % agarose gel. The gel was soaked with ethidium bromide. Fragments were visualized with an ultraviolet (UV) transilluminator, and the gel was photographed.

Bacterial DNA extraction from tomato tumors

Five protocols were tested to obtain DNA from tomato tumors for PCR analysis. The samples used in this test were made from the tumors obtained during the production of symptoms on tomato plants. Firstly, the tumors were washed in running water and tumor fragment surface were disinfected with 70 % ethanol. The necrotic tissues were removed; and tumor was cut in small fragment. The pieces of tumor were ground in 2 µl of sterile distilled water using a mortar and pestle to isolate the pathogen from plant tissues. After incubation during 30min, the macerates were filtered using sterile filter paper.

The first protocol used in this study to extract the DNA from the macerates was been described by Bereswill et al. (1992). The macerates were shaken for two hours in a 0.9% NaCl solution. The supernatant was centrifuged during 10min at 10000g, the pellet was resuspended in 0.1ml of sterile water, and 10µl was used for PCR-pTi analysis.

The second protocol was described in the study work of Llop et al. (1999). 500µl of macerates were placed into an Eppendorf and centrifuged at 10000g for 10min. The pellet was resuspended in 500µl extraction buffer (200 mM Tris HCL pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS, 2% PVP), vortexed and left for 1 hour at room temperature with continuous shaking. Then it was centrifuged at 5000g for 5min, 450 µl of the supernatant were taken 450µl isopropanol added, mixed and left for 1 hour at room temperature. The mixture was centrifuged and the pellet dried under vacuum. Finally, it was responded in 100 µl water.

For the third protocol, we use the modified extraction technic by Taylor et al. (2001). 500µl of macerates were placed into an Eppendorf and centrifuged at 10000g for 10min. the pellet was suspended in 500 µl of plant extraction buffer (140 mM NCL, 50 mM KCl, 0.05% Tween 20, 2% PVP and 0.4% BSA). The mixture was left at room temperature during 15min and stored in 4°C until use.

The fourth technic tested in this study was described in the study work of Szegedi and Bottka (2002). 1ml of the macerate was placed in Eppendorf and centrifuged at 18000g in 4°C for 15 min. the pellet was suspended in 100µl of sterile water in mixture with Triton X-100 (1%) (V:V) and heated at 95°C for 10 min.

The last protocol tested in this study was the same protocol described previously but with some modifications. The Triton X-100 solution was replaced with Tween 20 (0.1%). This protocol is based on the work of Bini et al. (2008).

PCR analysis

For all extraction protocols from tumors, PCR analysis was made using specific primers of *VirC1* and *VirC2* gens (VCF3 and VCR3). The reactional mix was prepared separately for each protocol (Table 1). PCR program was performed with the same condition as before.

Determination of the detection threshold of *A. vitis*

In order to evaluate the sensitivity of detection protocols of *A. vitis* by Bio-PCR, two bacterial suspension of 10⁹ CFU/ml were prepared, one in sterile water and the other in lysis solution of NaOH (20 mM). From the two bacterial suspensions, serial dilutions were made to a concentration of 10²CFU/ml. The detection threshold of *A. vitis* was also made for the macerates obtained from the previous tests. The PCR was performed using specific

primers VCF3 and VCF3 and according to the same condition as before.

III. RESULTS & DISCUSSION

Pathogenicity and hypersensitivity tests

A. vitis strain S4 was tested for the ability to induce hypersensitive response (HR) on tobacco and tumors in tomato. The tested strain is capable to cause HR in leaf of tobacco after 2 days of inoculation (Figure 1A) and able to induce tumor on stems of inoculated tomato within 20 days of incubation in greenhouse (Figure 1B). The capacity of this strain to induce gall development in tomato is related to the presence of pTi plasmid, which is an important element for the pathogenicity and tumorigenicity of *A. vitis* strains (Shams et al., 2012). The necrosis in leaf of tobacco results in rapid cell death and it is a part of the plant defense response against pathogens (Rodriguez-Palenzuela et al., 1991, Heath, 1998). The HR induced in leaf of tobacco it resembles a disease reaction in grape tissues infected with *A. vitis* in natural conditions. It has been demonstrated that several plant pathogenic bacteria that cause necrosis on host plant are able to induce HR to another plants (Alfano and Collmer, 1996). The ability of *A. vitis* to induce HR is due to the capacity of this strain to produce specific enzyme, Polygalacturonase (PG) (Rodriguez-Palenzuela et al., 1991). Other research have demonstrated that when the concentration of *A. vitis* greater than approximately 10⁶ CFU/ml, the *A. vitis* was able to cause necrosis (Herlache, 1999).

Bio PCR-pTi

The effectiveness of three protocols of the DNA extraction and two others methods without the extraction of the DNA, used for detection of *A. vitis* S4 from pure culture, was evaluated by molecular test with the primers VCF3/VCR3 (Figure 2). The results show that a 414 bp fragment of *virC1-virC2* gene (Sawada and Tsuchiya, 2003) was amplified with all the tested protocols of DNA extraction and without extraction using only a colony or a bacterial suspension; therefore, these protocols can be used for the different molecular tests to characterize and detect *A. vitis* strains. Moreover, the use of *virC* primers are able to detect pathogenic strains of *A. vitis* possessing pTi plasmid (Kawaguchi, 2009; Kumagai and Fabritius, 2008); and were used in several study work to identify tumorigenic strain of *A. vitis* in several countries (Kuzmanovic and al., 2012; Lamovšek et al., 2014).

Detection of *A. vitis* S4 from tomato tumors

The detection of *A. vitis* directly from infected plant represents an important technique for the diagnostic of the disease. In this work, five different protocols were used to extract the DNA from tumors produced in tomato plants.

Moreover, to compare these protocols the macerate, the enriched macerate with the PBS and pure colonies were used as a DNA template. The obtained results show the presence an expected size amplicon (414 bp) which amplified using the protocols 3 (Taylor et al., 2001), 4 (Szegedi and Bottka, 2002) and 5 (Bini et al. 2008) of extraction of DNA from plant tissues and also from enriched macerate (Figure 3). However, we cannot detect *A. vitis* with use of the protocol 1 (Bereswill et al., 1992) and 2 (Llop et al., 1999) of DNA extraction from infected tomato and also the direct detection from pure macerate.

The three protocols validated for the detection of *A. vitis* directly from plant tissues are based on the use of a buffer of extraction (Tween 20, the polyvinylpyrrolidone and bovine serum albumin) or only the detergent (Triton X-100 and Tween 20). These compounds can eliminate the inhibitors of PCR and also can cause lysis of bacterial cells. Plant compounds and in particularly the polyphenols and polysaccharides (De Boer et al., 1995) may limit the amplification of the bacterial DNA fragment extracted from plant material. Therefore, in the presence of these compounds, several authors suggest that the DNA should be purified using 2-mercaptoethanol or polyvinylpyrrolidone or by the use of commercial kits (Bereswill et al., 1992; Eastwell et al., 1995; Cubero et al., 1999; Taylor et al., 2001).

In the research work of Eastwell et al. (1995) they found that the bacterial lysis cells *in situ* in grapevine followed by DNA purification was more effective to detect *A. vitis* than then analysis of bacteria suspended in water. This observation may be due to the fixation of the bacteria to the cell walls of the grapevine cell. Moreover, in the study work of Kaufmann et al. (1996) they showed that the use of immunocapture culture of plant extract improve the sensibility and reliability of the method.

Determination of the detection threshold of *A. vitis*

For a large application of PCR for the detection of *A. vitis*, it is necessary to develop a rapid and simple protocol for the extraction of DNA for amplification and in the same time, the protocol must be sensitive for the detection of bacteria on low concentrations. For this reason, an evaluation test of the detection threshold of each protocol (from pure culture and macerate) was made to know the minimum concentration detectable by various protocols applied to the *A. vitis* strain. Different concentrations of DNA ranging from 10^9 CFU/ml to 10^2 CFU/ml were test for the evolution of the detection threshold.

The determination of detection threshold by Bio-PCR of different protocols of DNA extraction indicate that the three protocols of lysis cell from pure culture show different results. For the alkaline method of DNA extraction, the detection threshold was determinate in a

concentration equal to 5.10^4 CFU/ml (figure 4A). For the protocol of heating bacterial suspension, the detection threshold correspond to the concentration equal to 5.10^6 CFU/ml (Figure 4B). Moreover, for the third protocol of thermal shock the detection threshold correspond to the concentration 10^5 CFU/ml (Figure 4C). From the three tested protocols, the alkaline method was the more sensitive method for detection of *A. vitis* from pure culture. The alkalinity and high temperature cause the lysis of well cells and therefore the liberation of the DNA in the solution. The sensitivity of alkaline protocol can be explained by the additional NaOH property of the hydrogen bond perturbation between the DNA base pairs, which denatures genomic and plasmid DNA and allow their amplification. However the alkaline method is only used for the pure culture and isolated bacterial cells and cannot be used for the detection of *A. vitis* from plant material. In the study work of Burr et al. (1999) and Cubero et al. (1999) the detection threshold was determinate for 150 to 200 cells. Szegedi and Bottka (2002) were demonstrated that the detection threshold is equal to 10^5 CFU/ml using the protocol of heating bacterial suspension in sterile distilled water and in Triton X-100. The Triton X-100 solution was more effective than sterile distilled water.

For the determination of detection threshold, the protocol 4 and 5 used for the extraction of DNA of *A. vitis* from plant material and based on the use of Triton X-100 and Tween 20 were selected due to the simplicity and rapidity of these techniques. Therefore, it is necessary to determinate their sensitivity in order to identify their detection threshold. The sensitivity evaluation test of each protocol was carried using a decimal dilution of the macerate solution. The obtained results of specific PCR-pTi show that tested protocols are able to detect bacteria with concentration equal to 10^3 CFU/ml (Figure 5). These techniques can identify *A. vitis* with a low concentration from macerate that contain different bacterial species.

IV. CONCLUSION

In conclusion, results obtained in the present work show that use of alkaline method for the extraction of DNA from pure culture are the reliable and sensitive method can be used in several study for molecular characterization and detection of *A. vitis* isolates. Moreover, the use of direct techniques to detect *A. vitis* from plant materials by specific PCR are important issues that can be used for the detection of the pathogens from symptomatic and asymptomatic grapevines.

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Table.1: Reactional mix preparation for the different detection protocols of *A. vitis* directly from plant tissues

Protocol	Volume	Reactional mix
1	50	<ul style="list-style-type: none"> - 24.5 μl ultrapure water containing: BSA, 160μg/ml, (NH₄)₂SO₄, 16 mM and 2-mercaptoethanol, 10 mM. - 10 μl of DNA extract - 10 μl PCR buffer\times10 (Bioline) - 2.5 μl dimethyl sulfoxide (DMSO) - 1μl of each primer - 0.5 μl Tween 20 (100%) - 0.5 μl Taq polymerase (5 U)
2	50 μ l	<ul style="list-style-type: none"> - 29.76 μl ultrapure water - 10 μl PCR buffer\times10 (Bioline) - 5 μl of DNA extract - 2 μl Formamid - 1.32 μl of each primer - 0.6 μl Taq polymetrace
3, 4 and 5	20 μ l	<ul style="list-style-type: none"> - 9.8 μl ultrapure water - 4 μl PCR buffer\times10 (Bioline) - 5 μl of macerate - 0.5 μl of each primer - 0.2 μl Taq polymerase

From colony of <i>A. vitis</i> S4	20 μ l	<ul style="list-style-type: none"> - 13.8 μl ultrapure water - 4 μl PCR buffer\times10 (Bioline) - 1samll colony - 0.5 μl from each primer - 0.2 μl Taq polymerase
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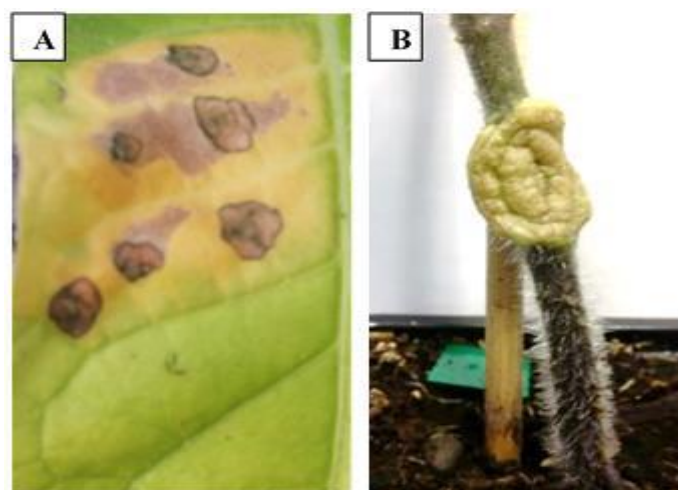


Fig.1: Hypersensibility and pathogenicity tests of *A. vitis* S4. **A:** hypersensitive response on tobacco leaf, **B:** development of tumor in the stem of tomato

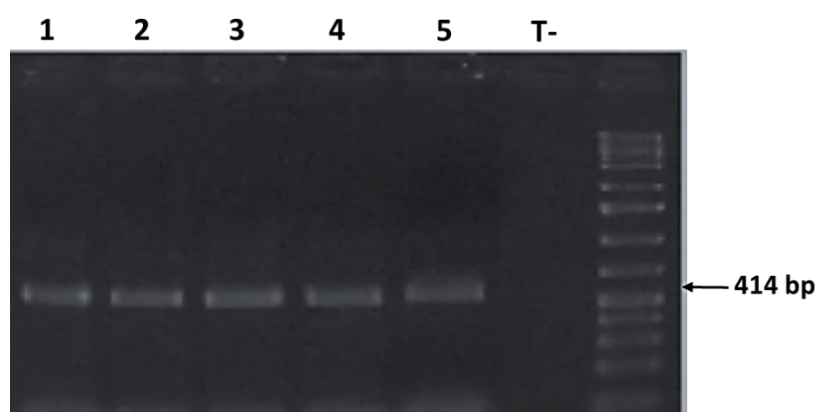


Fig.2: Electrophoretic profile of *A. vitis* S4 amplified with VCF3/VCR3 primers and using different protocols of DNA extraction from pure culture. **1:** bacterial colony, **2:** bacterial suspension, **3, 4 and 5:** DNA extraction from pure culture using protocols 1, 2 and 3 respectively.

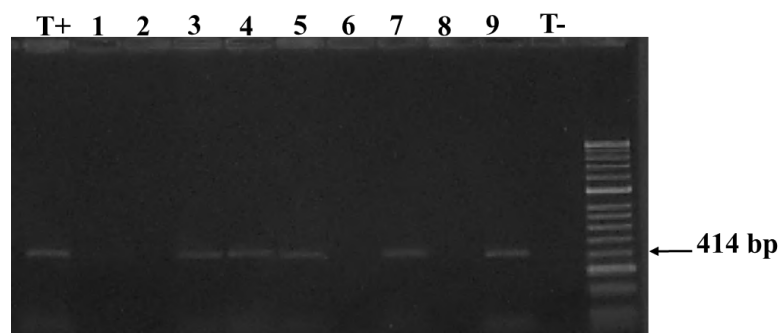


Fig.3: Electrophoretic profile of *A. vitis* S4 amplified with VCF3/VCR3 primers and using different protocols of DNA extraction directly from plant material (tomato tumors).

T+: positive control representing by extracted DNA from pure culture; **1, 2, 3, 4 and 5:** bacterial DNA extract from plant material using protocols 1, 2, 3, 4 and 5 respectively; **6:** pure macerate; **7:** enriched macerate; **8 and 9:** isolated colony from culture medium; **T-;** negative control.

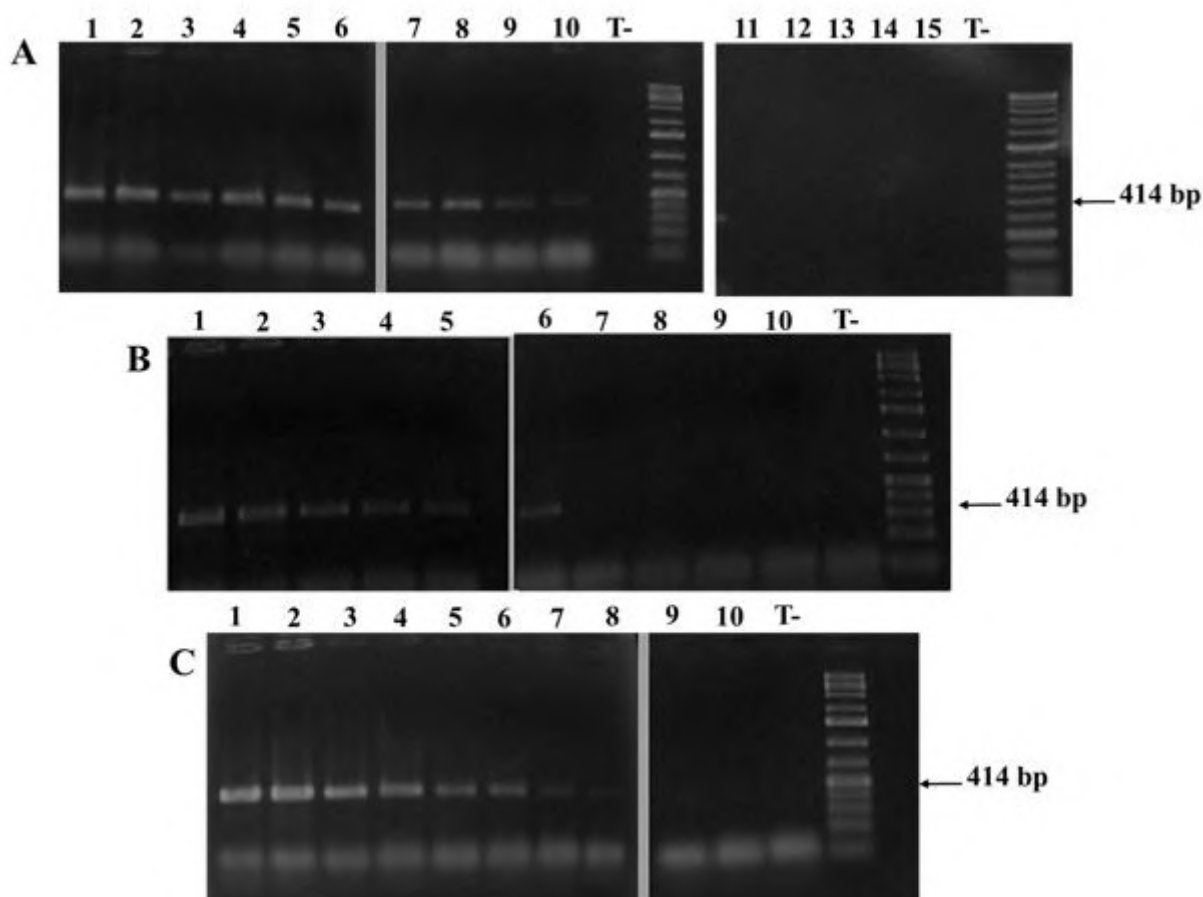


Fig.4: Evaluation of the sensitivity of different protocols of DNA extraction from pure culture. **A:** DNA extraction with alkaline method; **B:** DNA extraction with heating bacterial suspension; **C:** DNA extraction using thermal shock. From 1 to 15: different concentration of bacteria (CFU/ml). 1: 10^9 ; 2: 5.10^8 ; 3: 10^8 ; 4: 5.10^7 ; 5: 10^7 ; 6: 5.10^6 ; 7: 10^6 ; 8: 5.10^5 ; 9: 10^5 ; 10: 5.10^4 ; 11: 10^4 ; 12: 5.10^3 ; 13: 10^3 ; 14: 5.10^2 ; 15: 10^2 ; T-: negative control.

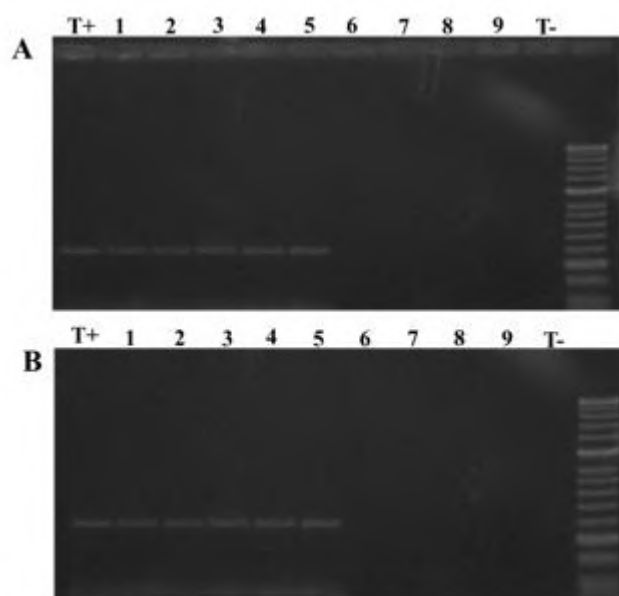


Fig.5: Evaluation of the sensitivity of different protocols of DNA extraction from plant material using, **A:** protocol 4 of DNA extraction with Triton X-100 and **B:** protocol 5 of DNA extraction with Tween 20. T+ : positive control ; 1 : 10^7 CFU/ml ; 2 : 10^6 CFU/ml ; 3 : 10^5 CFU/ml ; 4 : 10^4 CFU/ml ; 5 : 10^3 CFU/ml ; 6 : 10^2 CFU/ml ; 7 : 10^1 CFU/ml ; 8 : 10^0 CFU/ml ; 9 : 10^{-1} CFU/ml ; T- : negative control.