

## A Study on the Reaction of Some Grapevine Varieties to Crown Gall Agent (*Agrobacterium vitis*)

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### ABSTRACT

Grapevine is one of the oldest and most economically fruit crops. Grapes are a rich source of vitamins A, C, B6, as well as essential minerals, such as potassium, calcium, iron, phosphorus, magnesium and selenium. Crown gall disease (*Agrobacterium vitis*) is an economical disease in most vine yards. Since the bacterium remains in soil for a long time control of *Agrobacterium* is very difficult. Use of resistant rootstocks is the most effective methods for control of soil-borne pathogens, especially this bacterium. In this study, the reaction of the eight grape varieties (Shahani, Askari, Rish baba, Sefid-e-yaghuti, Qazvin Sefid-e-Keshmeshi, Qazvin Ghermez-e-Keshmeshi, Mehre and Rotabi) to crown gall was studied. In the first experiment, the rooted cuttings of different varieties inoculated in four sections with 20 ml of  $10^8$  cfu *A. vitis* and distilled water were compared as factorial experiment in a completely randomized design with four replications in green house. In the second set, inoculations were made by adding 40 ml of the suspension of two strains of bacteria (with the same concentration) around the root. Evaluations were made by growth and pathogenicity indices after five months. The callus formation on shoots was also studied in MS medium with and without bacterium. The results indicated that no varieties were immune to crown gall. Analysis of variance and mean comparisons of growth, physiological and pathogenicity indices showed the significant reduction in dry and wet weight of shoots and photosynthetic pigments in Shahni, sefid-e-yaquti and Rotabi. The soluble carbohydrate and anthocyanin also increased in these varieties. The highest necrosis, callus and gall formation were observed in Sefid-e-Yaghuti. It can be concluded that the Shahani, Rotabi and Sefid-e-Yaghuti varieties are most susceptible to causal agent of crown gall.

**Keywords:** *Agrobacterium vitis*; Crown gall

### INTRODUCTION

Crown gall is a serious disease of grapevines worldwide and is particularly severe in region where vineyards undergo freeze injury [1]. When crown gall is intense, vine vigor and productivity e significantly decrease [2] leading to considerable mortality of young vines in cold winter regions [3]. The strains of *Agrobacterium* associated with most grape crown galls are separate from other crown gall pathogens and had been grouped together as *Agrobacterium tumefaciens* (AT), biovar 3 [4]. They are now distinguished as *Agrobacterium vitis* (AV), separated from other *Agrobacterium* species by differences in DNA homology and metabolic characteristics [5]. Since crown gall-susceptible vines

seemingly allow *A. vitis* entry through roots [6], the use of resistant scion may decrease infection from soil inoculum and forbid subsequent infection of *A. vitis* free but highly crown gall-susceptible rootstocks cultivars.

Resistance to crown gall in *vitis* was reported by many researchers. Szegedi et al. [7] reported that elections of three Asian *vitis* species, *V. amurensis*, *V. piasezkii*, and *V. flexuosa*, formed no galls when inoculated with strains of *A. vitis* from Hungary (Like these strains were tumorigenic on *V. vinifera*. Crown gall resistance of *V. amurens* is reported to be dominant in crosses with *V. vinifera* [7]. Stover et al. [8] inoculated 40 various scions with five *A. vitis* strains and found that P775 was immune

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while small galls formed on 3309C and 101-14 Mgt, among others, following inoculation with some of the strains studied.

Utilization of resistant rootstocks may reduce the incidence of crown gall when *A. vitis* free vines are planted on *A. vitis* polluted site. The purpose of this study was to determine the response of grape varieties of crown gall and to identify the rootstocks which are resistant to crown gall.

## MATERIALS AND METHODS

### Plant material

Eight grape varieties (i.e., Shahani, Askari, Rish baba, Sefid-e-yaghuti, Qazvin Sefid-e-Keshmeshi, Qazvin Ghermez-e-Keshmeshi, Mehre and Rotabi) were obtained from the Isfahan University of Technology's farm. Rootstocks were held in sandy sweet in the greenhouse until they were rooted. Young plants were inoculated with *A. vitis* after the appearance of brown periderm. Typically shoots were 30-40cm long at the time of inoculation. The sites of inoculation were in the green space node.

### *Agrobacterium* strain

*Agrobacterium vitis* strains were kept at -80°C in cryogenic storage medium.

### Isolation of *A. vitis*

Vineyards and nurseries of grapevine (*vitis* spp.) were inspected for crown gall occurrence in Chaharmahal va bakhtiyari in Iran at the spring and autumn seasons. Samples of newly developed galls from the infected plants were collected and transported to the laboratory. Plant samples were washed with running tap water to remove soil particles, surface sterilized by dipping into 0.5% v:v sodium hypochlorite for 2 min, and rinsed three times with sterile distilled water (SDW).

Small portions were aseptically removed from each sample and placed in few drops of SDW in a mortar and pestle for maceration. The resulting suspension was left to stand for 30 min, and then loop full of the gall suspensions were streaked on RS medium [9]. Inoculated plates were incubated at 28°C until bacterial growth developed. Colonies consistent with *A. vitis* after 5 days on RS medium were streaked on potato dextrose agar (PDA).

### Identification of *A. vitis*

**Tumorigenicity:** The ability of the bacterial isolates to induce galls was tested on stems of tomato (*Lycopersicon esculentum*). Inoculum was prepared by suspending a loop full of bacterial growth from a 28-hold PDA slant culture into 2 ml distilled water. Plants grown in a greenhouse were inoculated at 2-4 sites per internode by pricking through a drop of inoculum with a needle.

The pathogenicity was determined 30 days after inoculation based on gall formation at the site of pricking. Also ability of this bacterium to callus formation was determined by carrot (*Daucus carota*) disks. Fresh carrot root disks were sterilized and

inoculated by 30-40 ml bacterial suspension  $10^8$ cfu on a moist filter paper in a petri plate [10,11].

**Phenotypical characterization tests:** Some phenotypical and biochemical tests for the identification and differentiation of *A. vitis* from other species were made as mentioned below.

**Callus formation:** Young actively-growing shoots were removed from plants and surface sterilized in 0.5% sodium hypochlorite for 10 min and then rinsed three times in SDW. Exposed ends of explants were removed. Nodal explants were placed in MS (Murashige & Skoog) medium in culture jars without regulator growth. 5 µL of the *A. vitis* 339-26 suspension ( $10^8$  cfu/ml) were spread over the exposed cut surface of each explant. As a control internodes were placed on MS medium with 0.049 µM indole butyric acid (IBA). Culture jars were kept in dim light and explants were sampled after 2 weeks inoculation.

**Ability to induction grape necrosis:** The ability of isolates to cause necrosis was determined on grape shoot explants. Actively growing shoots from potted vines in the greenhouse were harvested, and then surface disinfected by submersion in 0.5% sodium hypochlorite for 10 min and then rinsing with sterile distilled water.

Internodal sections were cut and supported vertically in 1% water agar in Petri dishes. The exposed ends of the explants were inoculated with 2 µL of bacterial suspensions ( $10^8$  CFU/ml). Three explants were used as a control, one untreated, one treated with sterile distilled water and one treated with *A. tumefaciens*. The appearance of necrosis was recorded after 72 h.

**Inoculation technique:** Two days before inoculation, the strain were cultured onto potato dextrose agar (PDA) (Difco) and grown at 28°C. Colonies from PDA were suspended at about  $10^9$  colony forming units (cfu) per ml in sterile de-ionized water (SDW). 10 µL of bacterial suspension were used to 1 mm diameter holes drilled through shoot nodes with typically 4 sites in green tissue. Inoculated sites were covered with parafilm and plants were grown in the greenhouse. After five months, inoculated sites were scored for gall formation.

In the second set, inoculations were made by adding 40 ml of the suspension of two strains of bacteria (with the same concentration) around the root.

### Measurements of biochemical parameters

**Chlorophyll and carotenoids:** for extraction of chlorophyll, 0.1gr fresh leave placed in mortar. Then 10 ml of 80% acetone added to sample and ground with mortar and pestle. The extraction was centrifuged 10 min in 2700 rpm. Supernatant was transferred to cuvette and absorbance was read in a UV-VIS spectrophotometer at 3 wavelengths, 470, 647,663. The concentrations of chlorophyll a and b were calculated based on following equations:

1. Chla:  $12.25A_{663}-2.79A_{647}$
2. Chlb:  $21.5A_{647}-5.1A_{663}$
3. Car:  $(1000A_{470}-1.82Chla-85.02Chlb)/198$

**Soluble carbohydrate:** 0.1 g of leaves (dry weight) was used for soluble carbohydrates determination by phenol-sulfuric acid method [11].

**Anthocyanin:** for extraction of anthocyanin, 0.1 g fresh leave with 10 ml methanol and HCL (99/1) ground with mortar and pestle. The extraction was centrifuged 5 min in 5000 rpm. Supernatant was transferred to cuvette and absorbance was read in a UV-VIS spectrophotometer at 550 nm wavelengths. The concentrations of anthocyanin were calculated with the following equations:

$$4. \text{Concentrations of anthocyanin} = \frac{\text{the absorbance}}{33000}$$

**Statistical analysis**

Data was subjected to analysis of variance and means were separated using Fisher’s protected LSD. The statement regarding statistical significance indicates that means were different at the P=0.05 level.

**RESULTS**

**Tumorigenicity**

In this study the effect of bacterial isolates to induce galls was studied. Figure 1 shows gall formation on tomato and callus formation on carrot.



**Figure 1:** (A) Gall symptoms on tomato stems three weeks after inoculation with *A. vitis*. (B) Callus formation on carrot discs three weeks after inoculation with bacteria.

**Table 1:** Phenotypical characteristics of *Agrobacterium* isolates.

Test	<i>A. tumefaciens</i> (Control)	Isolate (2)	Isolate (1)
Gram reaction	-	-	-
YDC pigment	Yellow	Yellow	Yellow
Fluorescent pigment	-	-	-
O/F	O	O	O
Catalase	+	+	+

**Determination of phenotypical characterization**

Typical *Agrobacterium* colonies from tumors and plant sap were isolated on NA media. They appeared to be convex, smooth with distinct edges. Table 1 shows the phenotypical characteristics of these isolate.

**Callus formation**

The size of explants, the ratio of hormones (or bacteria) and the genotype are major factors for the callus formation of grapevines. In this study, genotype of grape was checked. According to the result, Rish baba and Qazvin Sefide-Keshmeshi have highest rate of callus in bacteria and hormone treatment (Figures 2 and 3).

Gelatin hydrolyses	-	-	-
Utilization of Malonic Acid	-	+	+
L-Tartaric Acid	-	+	+
Motility at PH 7	+	-	-
Pectolytic at PH 4.5	-	+	+

+: Positive reaction or growth

-: Negative reaction or growth



Figure 2: Callus formation in Yaghtuti variety. A: by *A. vitis*, B and C: by oxin hormone.

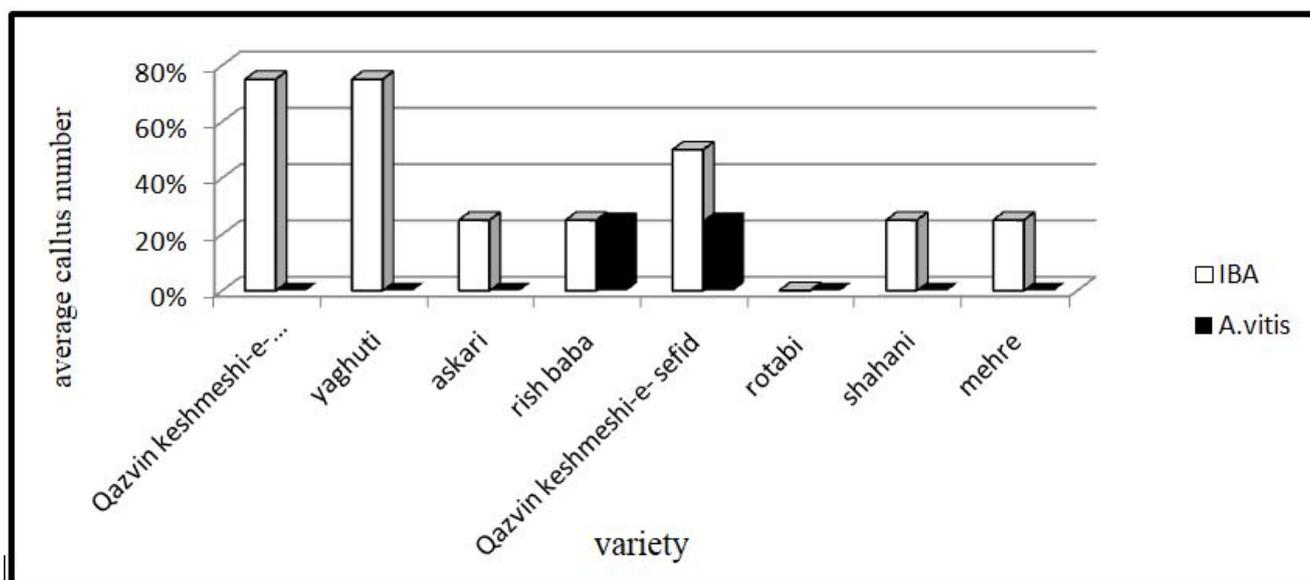


Figure 3: Average of callus formation in grape varieties by two treatments (IBA and *A. vitis*).

### Ability to induction grape necrosis

These isolates were identified that caused necrosis on grape explants (Figure 4). Severity of necrosis differed between varieties which bacterium caused a black necrosis with ooze within 48 h in Shahani and Yaghtuti. By streaking cut ends of the explants on NA medium, the standard colonies of *A. vitis* readily appeared after 2 days (Table 2).



Figure 4: Necrosis on grape explants (Yaghtuti) resulted from artificial inoculation by *A. vitis*.

Variety	Treatment	Necrosis (mm)	depth
Askari	<i>A. vitis</i>	1.37 <sup>abc</sup>	
Askari	<i>A. tumefaciens</i>	1.50 <sup>ab</sup>	
Askari	sterile distilled water	0.75 <sup>defg</sup>	
Askari	Control	1.00 <sup>bcde</sup>	
Qazvin Ghermez-e-Keshmeshi	<i>A. vitis</i>	0.50 <sup>efgh</sup>	
Qazvin Ghermez-e-Keshmeshi	<i>A. tumefaciens</i>	0.62 <sup>efgh</sup>	
Qazvin Ghermez-e-Keshmeshi	sterile distilled water	0.75 <sup>h</sup>	
Qazvin Ghermez-e-Keshmeshi	Control	0.15 <sup>h</sup>	
Qazvin Sefid-e-Keshmeshi	<i>A. vitis</i>	0.62 <sup>efgh</sup>	

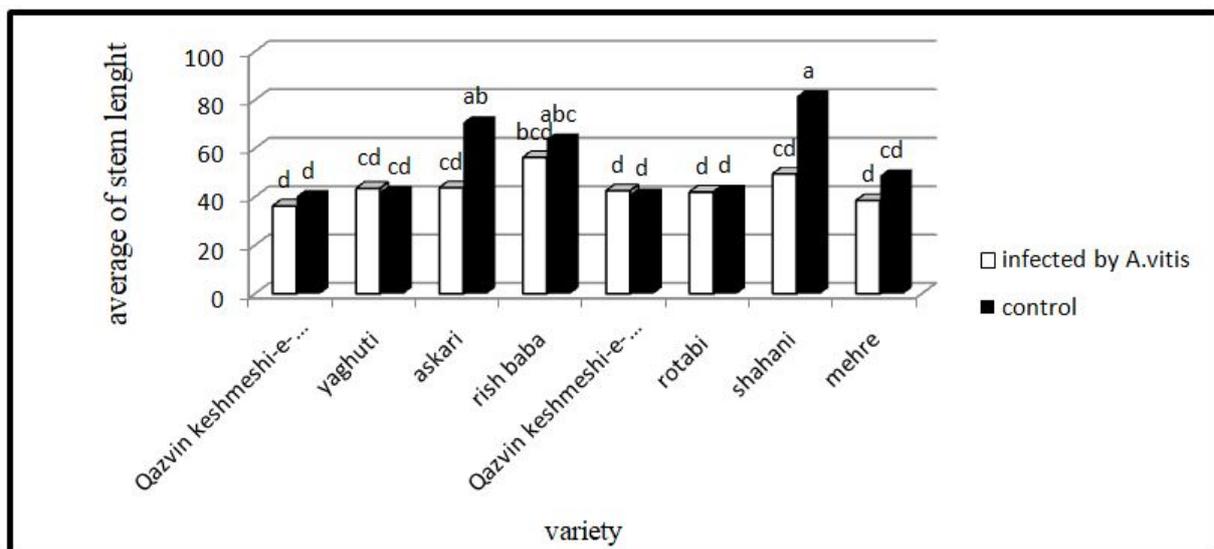
Qazvin Sefide-Keshmeshi	<i>A. tumefaciens</i>	0.75 <sup>defg</sup>
Qazvin Sefide-Keshmeshi	sterile distilled water	0.37 <sup>fgh</sup>
Qazvin Sefide-Keshmeshi	Control	0.25 <sup>gh</sup>
Mehre	<i>A. vitis</i>	1.75 <sup>a</sup>
Mehre	<i>A. tumefaciens</i>	1.25 <sup>abcd</sup>
Mehre	sterile distilled water	0.62 <sup>efgh</sup>
Mehre	Control	0.50 <sup>efgh</sup>
Rish baba	<i>A. vitis</i>	1.37 <sup>abc</sup>
Rish baba	<i>A. tumefaciens</i>	1.5 <sup>ab</sup>
Rish baba	sterile distilled water	0.52 <sup>efgh</sup>
Rish baba	Control	0.15 <sup>h</sup>
Rotabi	<i>A. vitis</i>	1.00 <sup>bcde</sup>
Rotabi	<i>A. tumefaciens</i>	0.87 <sup>cdef</sup>
Rotabi	sterile distilled water	0.27 <sup>gh</sup>
Rotabi	Control	0.15 <sup>h</sup>

Shahani	<i>A. vitis</i>	1.37 <sup>abc</sup>
Shahani	<i>A. tumefaciens</i>	0.87 <sup>cdef</sup>
Shahani	sterile distilled water	0.37 <sup>fgh</sup>
Shahani	Control	0.25 <sup>gh</sup>
Yaghuti	<i>A. vitis</i>	1.62 <sup>a</sup>
Yaghuti	<i>A. tumefaciens</i>	0.75 <sup>defg</sup>
Yaghuti	sterile distilled water	0.37 <sup>fgh</sup>
Yaghuti	Control	0.17 <sup>h</sup>

**Table 2:** Mean comparison\* of necrosis depth caused by crown gall agent (*A. vitis*).

**Plant growth indices**

Analysis variance of bacterium action on plant growth indices showed a significant effect (Figure 5). This bacterium reduced the growth vigor, especially stem length. Highest effect of this bacteria observed on Shahani variety. Tumors originate from dividing plant cells, e.g. from cambium. Thus the cambial region becomes unable to differentiate into efficient phloem and xylem elements leading to deficient nutrient transport, and grapevine decline, and vine death and yield reduce [12].



**Figure 5:** Mean comparisons\* of stem length in eight grape varieties inoculated by *A. vitis* \*Means with the same letters are not significantly different at 5% level according to LSD test.

**Plant biochemical indices**

**Chlorophyll and carotenoids:** Mean comparison of effect of bacterium on plant photosynthesis pigments showed different

action (Figure 6). According to these results, bacterium could reduce photosynthesis pigments, but most reduction observed by Yaghuti and Rish baba.

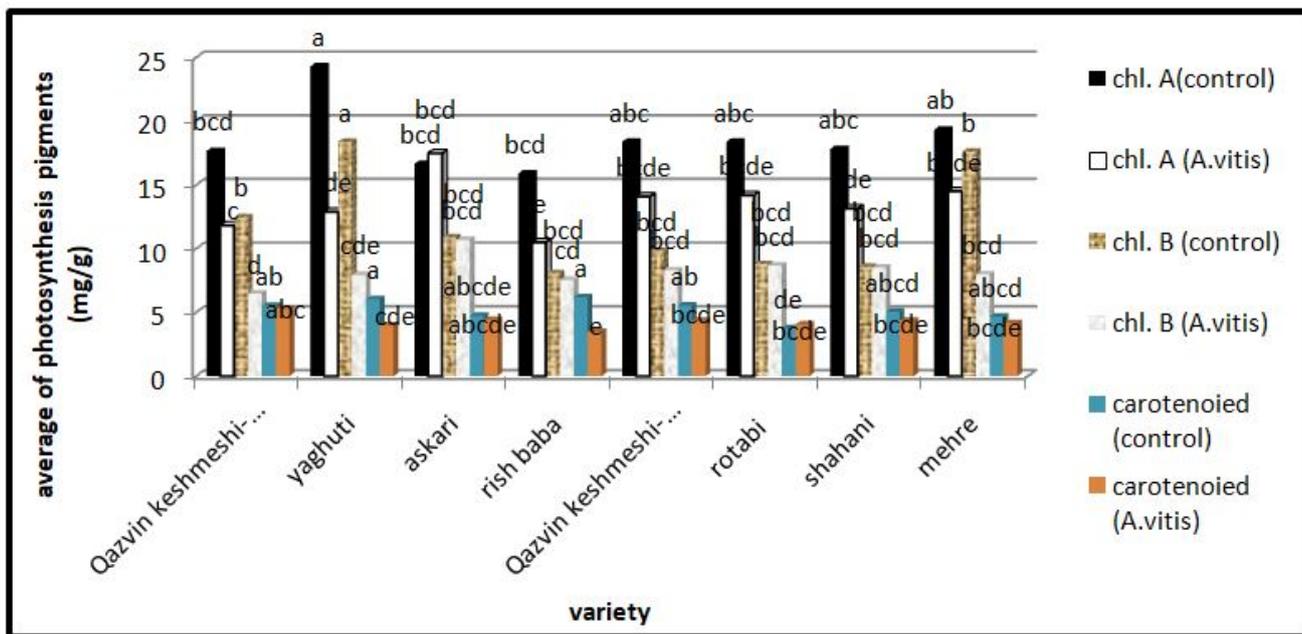


Figure 6: Mean comparisons\* of photosynthesis pigments in eight grape varieties inoculated by *A. vitis*; \*Means with the same letters are not significantly different at 5% level according to LSD test.

According to literature, drought is one of the leading environmental stresses decreasing plant photosynthesis pigments, then growth and productivity [13]. Gall formation on roots and shoots, leads to disturbance of absorption and transmission of water and finally lack of water and water stress in the plant occurs.

**Soluble carbohydrate:** The result of this study showed that the soluble carbohydrate in the leaves of treatment plants significantly increased. The greatest increase was observed in the Shahani and Rotabi (Figure 7).

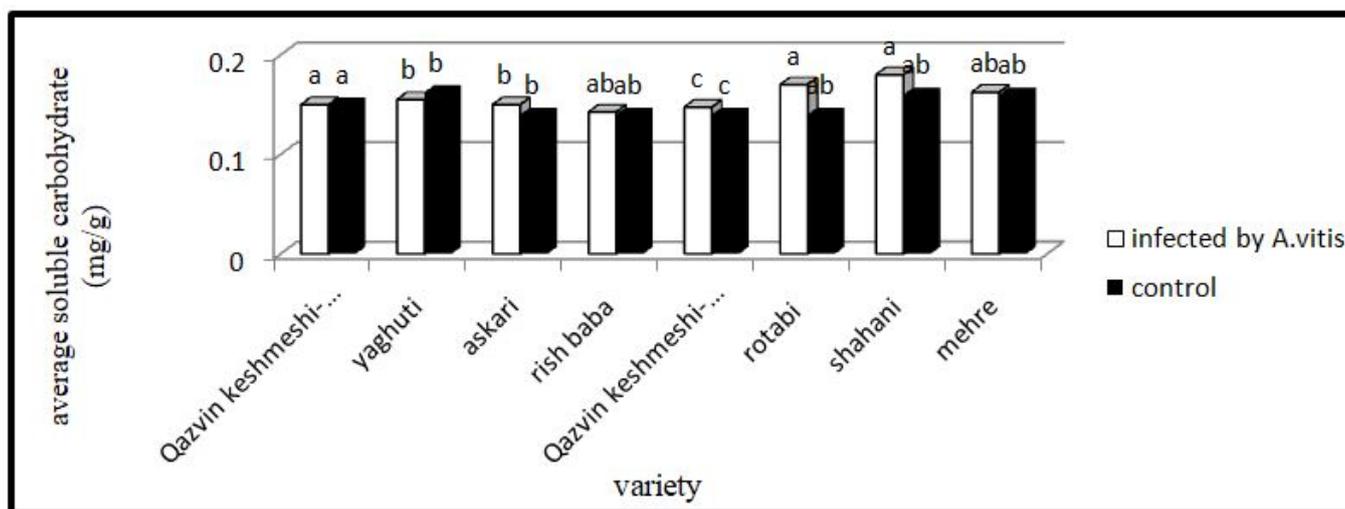
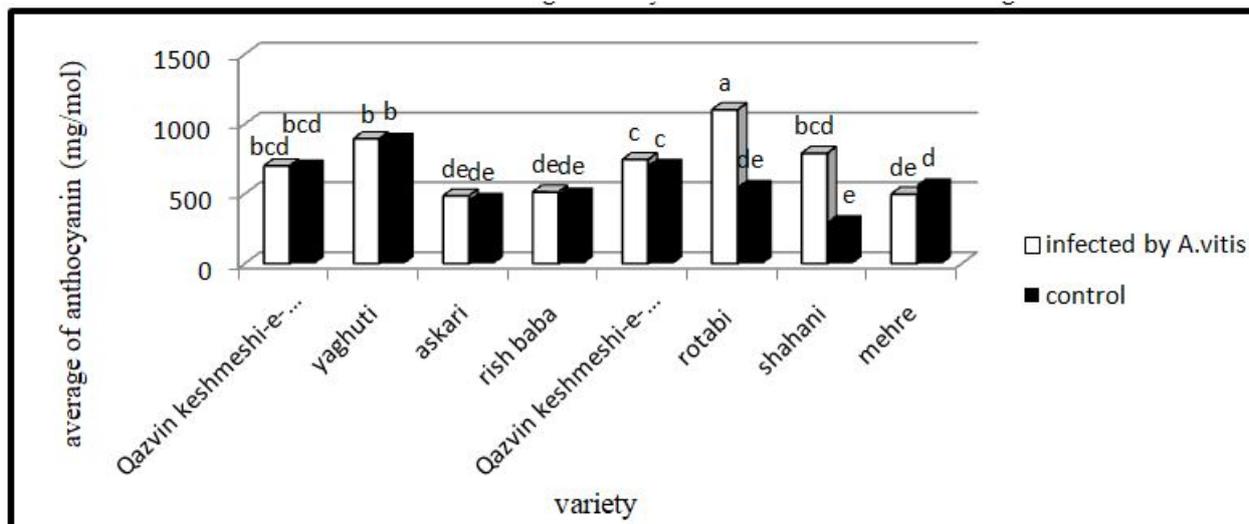


Figure 7: Mean comparisons\* of soluble carbohydrate in eight grape varieties inoculated by *A. vitis*; \*Means with the same letters are not significantly different at 5% level according to LSD test.

Studies show that stress increases soluble carbohydrate. Accumulation of Soluble carbohydrate in these condition helps to regulate intercellular osmolarity [14].

increased anthocyanin content in the plant. Treatment were infected with bacteria have more anthocyanin, while highest significant increase in Shahani and Rotabi was observed.

**Anthocyanin:** Mean comparison of effect of bacterium on plant anthocyanin showed different action (Figure 8). Bacteria



**Figure 8:** Mean comparisons\* of anthocyanin eight grape varieties inoculated by *A. vitis*; \*Means with the same letters are not significantly different at 5% level according to LSD test.

Accumulation of anthocyanin in leaves, shoots and root by several environmental stress such as drought and wound has been reported earlier [15]. In this study bacterial infection, drought and wound are most important factors increasing the amount of anthocyanin.

**Crown gall agent pathogenicity indices**

Analysis variance of bacterium pathogenicity indices showed no significant different between varieties (Table 3). Yaghuti has highest gall between eight varieties (Figure 9).

Tumors are a proliferation and agglomeration of tissue caused by the toxins secreted by bacteria. Cytological aspect of tumor tissue present within a tumor is much like that found in humans and animals. In a cross-section of tumor cells large, giant cells where are surrounded by normal-sized cells were observed. Many cells are polinuclate and retain strong dyes. In the tumor was found an accumulation of organo-forming substances (ex:  $\beta$ -indol acetic acid) and organic acids (malic, citric, oxalic, etc.) [16].

**Table 3:** Analysis variance of crown gall agent pathogenicity indices on eight infected grape varieties.

Sources of Variation	Df	MS (Mean Square)
		Gall number
Variety	7	7.45 <sup>n.s</sup>
Error	24	4.71 <sup>n.s</sup>
CV%		27.44

n.s.: No Significant at the 0.05 level



**Figure 9:** Gall formed on stem of Yaghuti by *A. vitis*.

**Crown gall agent pathogenicity indices (second set of experiment)**

Analysis variance of bacterium pathogenicity indices showed significant different between eight grape varieties. The number of gall in grape varieties was different. Yaghuti had the largest number of gall were infected with both isolates. Thus, Yaghuti was the most sensitive variety in this study. On the other hand, Shahani had the lowest number of gall which can be due to its insensitivity to growth hormone (Figures 10 and 11).

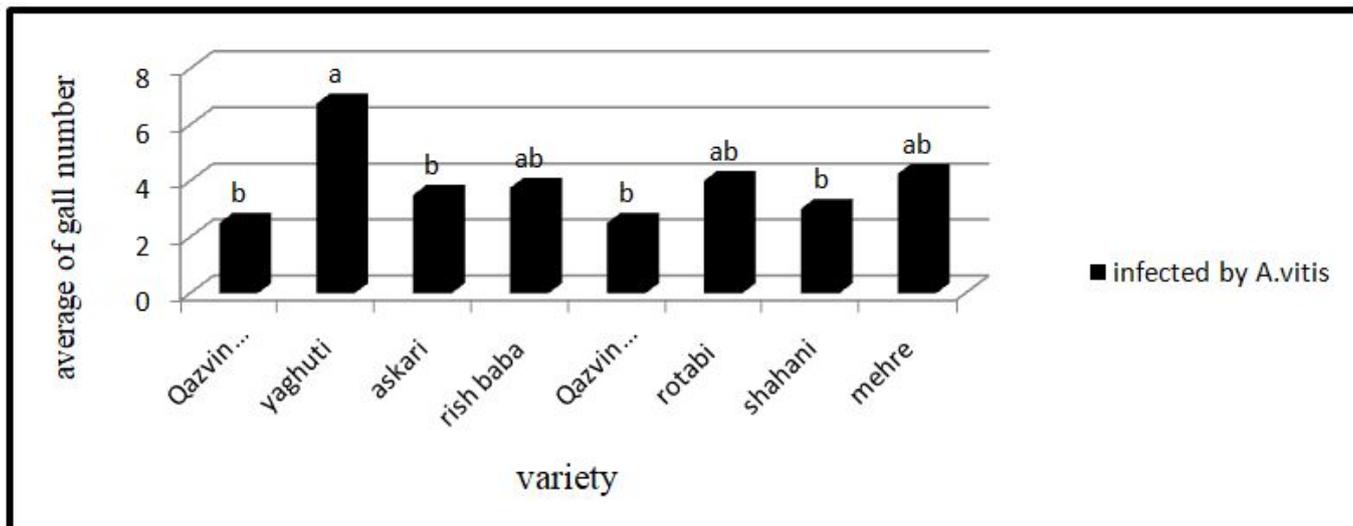


Figure 10: Mean comparisons\* of crown gall agent pathogenicity indices on eight infected grape varieties; \*Means with the same letters are not significantly different at 5% level according to LSD test.

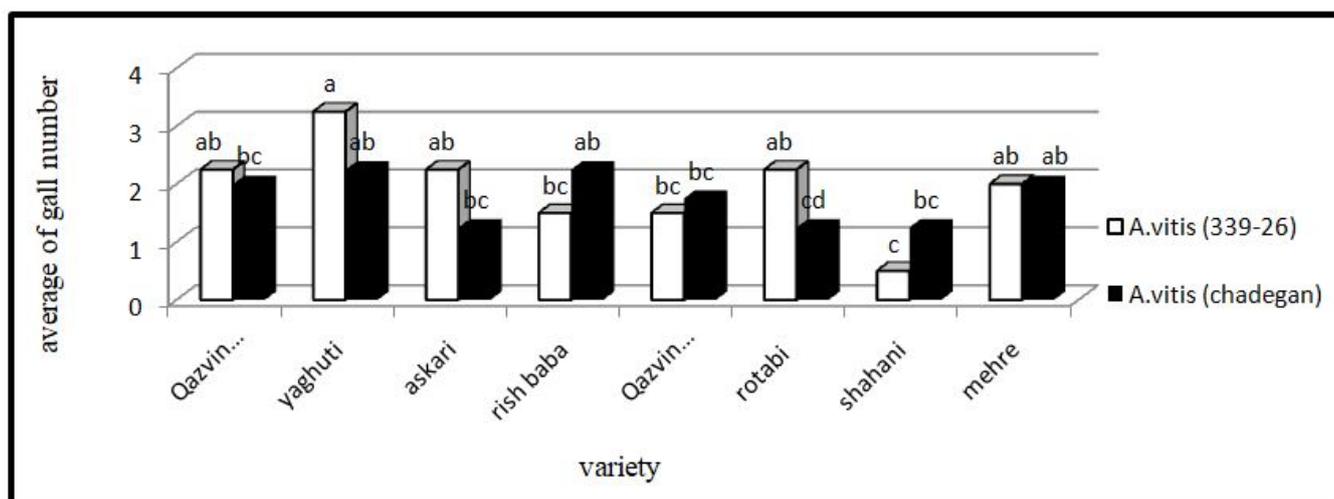


Figure 11: Mean comparisons\* crown gall agent pathogenicity indices of two isolates in eight grape varieties; \*Means with the same letters are not significantly different at 5% level according to LSD test.

DISCUSSION

In this study, no variety was found to be immune to crown gall, while response of different varieties to inoculation with *A. vitis* varied widely. In all grape varieties, gall formation leads to disruption of the vascular system. That finally led to diminishment growing plant. Highest reduction of growth indices observed on Shahani. The plant infection with bacteria (*A.vitis*) showed an influence on biochemical parameters. Shahani is most sensitive variety in biochemical parameters. Also callus formation and necrosis depth showed that Yaghuti is the most sensitive variety to *A. vitis*. Shahani was sensitive to *A. vitis*, and bacterial infection made reduction effect on growth indices and adequate to biochemical indices in this variety. While the number of gall formed was lowest among another varieties, that according to result of callus formation, Shahani is not susceptible to growth regulator hormones (auxin and cytokinin).

CONCLUSION

Finally according to these results, Shahani, Yaghuti and Rotabi are most sensitive varieties to crown gall agent. Rootstock resistance to crown gall may be important in preventing passage of soil *A. vitis* in to susceptible scions. However, other factors are probably involves in the development of systematic infections. Also freeze injury is an important factor in systemic *A. vitis* development in the field, and use of resistance rootstock to Freezing is an effective way to control of this dieses. When resistant rootstocks form galls, populations of *A. vitis* in the nodal tissues remain much lower than in susceptible and this may affect the extent of endophytic colonization. However, it is importance to note that high populations of *A. vitis* can survive at inoculated sites of both crown gall-resistant and susceptible *vitis* rootstocks for more than a year without inducing visible crown gall symptoms.

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