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Effect of Polarity on *in vitro* Tumor Formation by *Agrobacterium tumefaciens* and Necrotic Response of Grape Cultivars

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Abstract

Tumor formation by *Agrobacterium tumefaciens* (AT) was significantly increased when the grape stem explants were planted and inoculated basal end upwards. When stem pieces of Catawba and Chancellor cultivars of *Vitis vinifera* were inoculated basal end upwards, these exhibited a 40% increase in tumor occurrence and a 3-4 times increase in tumor size. The enhanced tumor production reflects basipetal auxin accumulation in the grape stem explants. This *in vitro* inoculation procedure provided an efficient method to study pathogenicity of AT strains on a large scale in grape cultivars.

Some necrosis accompanied tumor formation following *in vitro* inoculation in normal upwards direction. In some cases, tumors were produced in early stages but they became necrotic later. It might be possible that the higher auxin production in transformed cells lead to higher auxin oxidase activity which might be responsible for this necrosis.

Key words: Polarity, tumor formation, *Agrobacterium tumefaciens*, Necrotic response, Grape cultivars

Introduction

Crown gall, caused by *Agrobacterium tumefaciens* (AT), is a serious disease in many plants of economic importance. Generally it damages grapevines but *Vitis vinifera* (grapes) is severely damaged as compared to intraspecific hybrids and other *Vitis* spp. The pathogen induces tumor formation on roots and the disease becomes more acute when the aerial parts are affected. It, most commonly, engirdles the trunk and kills cold-sensitive cultivars (Burr, 1978). Panagopoulos *et al*. (1973, 1978) identified a new group of AT mainly from grapevine and it was characterized as biotype 3. Reports from other parts of the world, later, confirmed that the biotype 3 strains are predominant in grape-growing areas (Sule, 1978; Burr and Hurwitz, 1981; Loubser, 1978; Ma *et al*., 1987). Biotype 1 strains have also been found forming galls, but they are less prevalent (Burr and Katz, 1984; Sule, 1978). Classification of AT into three biotypes is chrornosomally determined (Kerr and Panagopoulos, 1977). In grape-growing areas, virulent bacteria are mainly present in the plants and their rhizosphere, while the majority of the soil isolates are avirulent (Burr and Katz, 1983).

Grape cultivars form tumors of variable size, depending on their sensitivity to crown gall. Some cultivars show small tumor formation while large tumors develop on certain sensitive cultivars where they limit transport in vascular system leading to ultimate death of the plant in a single season. Tumor development, however, appears to be a reflection of hormonal levels in the transformed cells. Cleveland and Goodman (1987) proposed that tumor size in grape cultivars might be a function of the inherent sensitivity of normal cells to be stimulated by phytohormones synthesized and excreted by adjacent transformed cells. They further suggested that large tumors might indicate higher level of phytohormone synthesis or greater number of transformed cells. The purpose of this study was to demonstrate the effect of polarity to the tumor formation or necrotic response of the grapes when inoculated/infected with AT.

Several methods have been developed to check the virulence of *Agrobacterium tumefaciens* artificially but in *Vitis* spp. there are few such reports. Hemstad and Reisch (1985) inoculated intact and decapitated grape stems and used either dipping the wounded surface in AT colonies or piercing the stem with needle and then applying the inoculum on wounded area. They found piercing inoculations more successful as compared to dipping method and that the decapitation had no significant effect on tumor development. Lowe (1985) used stem pieces with one lateral bud and stuck them basal end up on hormone free Murashige-Skoog (MS) medium. She inoculated the basal ends of the stems with loop full of AT strains. Significant induction of tumors were observed and concluded that polarity had a positive effect on the development of tumors as compared to normal shoot inoculations. The same method with some modifications has been adopted to further investigate the effect of polarity on tumor development and its size.

Materials and Methods

Plant material: Grape species, cultivars and hybrids were grown in the green house and apparently healthy and well growing plants were selected for all experiments. Young shoots with 4-6 internodes were harvested and kept in polyethylene sacks before use to retain their freshness. The plant material was used either immediately after harvesting or where this was not possible, it was stored at 4°C for a limited period of time.

Bacterial cultures: The bacterial strains of *Agrobacterium tumefaciens* used in these studies belonged to both biotype 1 and 3. These strains are summarized in Table 1 indicating their source of origin. All strains were maintained on either nutrient yeast dextrose agar (NYDA) (g/d: Nutrient agar 23, yeast extract 5, dextruse 5) or yeast extract sucrose medium (g/l: nutrient broth 5, Bacto peptone 5, sucrose 5, yeast extract 1, $MgSO_4.7H_20$ 0.5, agar 1 5) and incubated at 28°C. In all experiments, 24-48 h old cultures were used. Inocula were prepared in nutrient broth (NB) and the bacterial density was adjusted to 10^8 cells/ml as measured by a Beckman DB-G spectrophotometer.

Table 1: Tumorigenic strains of *Agrobacterium tumefaciens*

Strain	Biotype	Source
FACH	1	This laboratory*
FA1	1	This Laboratory*
A281	1	Nester, E.W. (University of Washington, Seattle, Washington, USA)
Ag63	3	Panagopoulos, C.G., Benaki Phytopathogenic Institute, Kiphissia, Athens, Greece.
Ag63s(Sr/Rif)R	1?	This Laboratory*
G-3	3	This Laboratory*

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Polarity effect: Tumor formation on grape explants was studied by planting them apex upward and compared with those planted basal side upward. The percentage of stems that formed tumors and their tumor size were measured since polarity plays an important role in tumor formation (Lowe, 1985). Furthermore, the inoculations at wounded nodal area and internodal sites were carried out to have a comparative account of tumor formations.

Tumor size measurement: Tumor size was measured on 21st day after inoculations. The tumors or callus like growths were excised from explants and their volumes were determined by water displacement method. Average volume (cm^3) of tumors for cultivars tested was calculated on the basis of inoculated stem surface (cm^2). Additionally, the incidence percentage of tumor formation by a specific cultivar was also determined.

Necrosis assay: In certain instances, explants showed necrosis at and around the inoculated surface which tends to progress downwards. The percentage incidence and severity were recorded for necrosis where it appeared. Severity was scored on the basis of necrotic lesion length along the axis of the explant.

Results

Effect of polarity was determined when stem explants were inoculated basal side up and compared to upside upwards inoculation. The former showed a significant increase in tumor formation. The data for tumor formation is summarized in Table 2. All 3 cultivars tested for tumor formation in this fashion, showed more than 20% increase in tumor formation. Catawba exhibited most significant effect where it was more than 80 percent.

Table 2: Effect of polarity on tumor formation by *Agrobacterium tumefaciens*

	AT (Strains)		
	Ag63s	FACH	FA1
% tumor formation #			
Chancellor			
Apical side upwards	18.9	25.0	3.1
Basal side upwards	56.0	83.3	79.2
Catawba			
Apical side upwards	2.9	3.2	6.5
Basal side upwards	82.1	91.7	100.0
Seyval Blanc			
Apical side upwards	55.6	33.3	n.d
Basal side upwards	87.5	37.5*	87.5

*Tumors formed initially but showed necrosis at the time of data taken; n.d. not determined; #Data represent mean of three experiments where each experiment contained 10 shoots which were inoculated for each treatment.

The polarity effect was also prominent in case of tumor size measurement (Table 3). In all three cultivars i.e., Chancellor, Seyval Blanc and Catawba showed increase of more than three folds with all three inoculated strains of *Agrobacterium tumefaciens*.

Table 3: Effect of polarity on tumor size caused by *Agrobacterium tumefaciens*

	AT (Strains)		
	Ag63s	FACH	FA1
Tumor size*, #(cubic centimetre/square centimetre)			
Seyval Blanc			
Apical side upwards	0.23	0.64	0.60
Basal side upwards	0.55	0.64	0.60
Chancellor			
Apical side upwards	0.23	0.12	0.00
Basal side upwards	0.33	0.59	0.39
Catawba			
Apical side upwards	0.13	0.10	0.10
Basal side upwards	0.46	0.41	0.34

*Tumors size measurements indicate the control measurements substraction; #Data represent mean of three experiments where each experiment was consisted of at least 10 inoculations per treatment.

Table 4: Percentage occurrence and severity of necrosis caused by *Agrobacterium tumefaciens*

	AT (Strains)				
	Ag63s	FACH	FA1	G-3	A-281
	Percentage necrosis				
Vitis vinifera					
Chancellor					
OCC*	37.3	27.6	24.0	46.4	63.5
Sev**	23.1	21.4	26.3	59.5	22.0
Catawba					
OCC*	52.5	0.00	0.00	33.0	30.0
Sev**	70.0	0.00	0.00	n.d.	0.00
Seyval Blanc					
OCC*	16.3	0.00	12.5	62.5	36.0
Sev**	35.0	0.00	90.0	37.4	42.0
V. cinerea					
OCC*	58.4	80.0	65.0	66.7	66.7
Sev**	60.0	21.2	32.5	n.d.	n.d.

*Percentage necrosis occurrence; **Percentage necrosis severity; n.d. not determined; #Data represent the mean of three replicates.

Table 5: Effect of polarity on induction of necrosis (% necrosis) caused by *Agrobacterium tumefaciens*

	AT (Strains)			
	Ag63s	FACH	FA1	G-3
% Necrosis*				
Grape Cultivars				
Chancellor				
Apical side	69.7	16.7	23.6	38.8
Basal side upwards	42.3	16.2	16.7	25.0
Catawba				
Apical side	41.3	14.2	42.9	n.d.
Basal side upwards	0.00	0.00	0.00	n.d.

n.d. not determined; *Data represent the mean of three experiments.

Necrosis: When *in vitro* inoculation of some of the explants with *Agrobacterium tumefaciens* were carried out, necrosis accompanied with tumors formation but sometime it proceeded alone instead of tumor formation. The necrosis proceeded from the upper inoculated surface downwards. Neither its occurrence nor its severity (Table 4) were consistently detected in a particular grape cultivar. All cultivars whether sensitive or less sensitive to crown gall, exhibited necrosis to some extent.

Necrosis varied in its appearance. The most common form of necrosis observed was a dried necrotic area that developed at the inoculated surface of stem explant and revealed no signs of tumors formation at any stage. It appeared soon after inoculations, normally within 10 days. However, in some cases, the inoculated area first showed tumor development but later on it became watery. Plant decay which started at the inoculated surface, proceeded downward showing some oozing at the top. Moreover, biotype 3 strains isolated from grapes were observed to cause more necrosis when compared with biotype 1 strains. *Agrobacterium tumefaciens* strains Ag63s and G3 both were more necrogenic than FACH and FA1 (Table 5). *Vitis cinerea*, a resistant species to *Agrobacterium tumefaciens*, showed highest necrosis.

Discussion

The level of indole acetic acid (IAA) increases in the basal portions of the grape cuttings and it remains high at least for 24 hours (Moncousin *et al*., 1989). This supported our findings when we inoculated basal ends (nodal site) with *Agrobacterium tumefaciens* and placed upside downwards in the agar medium, it increases both incidence and severity of tumor development.

The higher endogenous level of IAA at the time of inoculations in wounded area may be the reason for enhanced *Agrobacterium tumefaciens* infection. Polarity effect is further supported by the studies of Lowe (1985) who used grape cuttings for *in vitro Agrobacterium tumefaciens* inoculations. It is also possible that node area provides larger and less woody surface for *Agrobacterium tumefaciens* infections as compared to normal internode surface (Table 2). This effect is more pronounced in Catawba because its shoots are comparatively thin and woody which provides less number of cells for *Agrobacterium tumefaciens* infection development. However, when direction is reversed and basal end is inoculated at node area, it provides larger and softer tissue for *Agrobacterium tumefaciens* infectious.

Polarity appeared to affect the development of necrosis since basal side up inoculations of *Agrobacterium tumefaciens* strains lead to decrease incidence (occurrence) of necrosis (Table 5). Catawba responded better and displayed a 100 percent tumor formation in all experiments done to study polarity effect. This was an interesting situation that all these experiments which were repeated three times, gave the same response in a single growing season when the plants were growing very well vegetatively. Later on, necrosis caused by *Agrobacterium tumefaciens* strains were observed in grape cultivars (Table 5). This variability in response might be a result of some environmental factors which influence the growth pattern of a plant at different time periods of a year.

The grape cuttings showed higher ethylene production at both ends of the grapevine cuttings (Moncousin *et al*., 1989). Auxin accumulation is confined to basal end. Ethylene may lead to higher activity of peroxidases which, in return oxidize polyphenols. This higher peroxidase activity and the generation of phenolic compounds may result in the death of inoculated cells and bacterial inoculations may exacerbate this effect which results in the development of severe necrotic symptoms. The necrosis which appeared less at basal end may be due to accumulation of IAA which increases the metabolic activity, for example permeability, in the inoculated cells. It is

possible that ethylene itself may be a cause of necrosis at upper end while its effect is minimized by auxin at basal end. The necrosis is some time named as hypersensitivity (Lowe, 1985). This phenomenon was observed in the resistant grape variety 'Steuben' by Lowe (1985) and suggested that it might be due to triggering of host defense mechanism. It is quite possible that necrosis is a host response to *Agrobacterium tumefaciens* infection, but it is also observed in highly susceptible cultivars like Chancellor and Seyval Blanc. Near to us it is a host response in highly susceptible cultivars like Chancellor and Seyval Blanc. Near to us it is a host response which results in the activation of host defense mechanism, though we cannot confine it to only insensitive cultivars. Furthermore, it is morphologically variable, it is hard to draw a line between necrosis and hypersensitive reaction in this host parasite interaction. The necrosis exhibited by resistant cultivars is termed as hypersensitivity on the basis of incompatible interaction. Rest of the necrosis, may also be a form of hypersensitive reaction. We also observed necrosis in those cases where first a small tumor developed which progressed to necrosis, which might be due to excessive evaluation of ethylene which killed the tumors as well as its surrounding areas.

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