Egypt. J. Agric. Res., 84 (3), 2006

INDUCED SYSTEMIC RESISTANCE AGAINST *AGROBACTERIUM TUMEFACIENS* BY CERTAIN BIOTIC AND ABIOTIC AGENTS

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(Manuscript Received 6 September 2005)

Abstract

Under greenhouse conditions, control of crown gall disease was tried through systemic resistance induced by organic acids, salts and bacteria. Oxalic and salicylic acids, mono-and dibasic sodium phosphate as well as *Bacillus subtilis* and *Pseudomonas fluorescens* were used. The afore-mentioned inducers were evaluated for induced systemic resistance (ISR) in *Prunus persica* seedlings against *Agrobacterium tumefaciens*. The inducers were sprayed on peach leaves followed by inoculation with the pathogen. Salicylic acid was highly effective in controlling the disease followed by *P. fluorescens*, monobasic potassium phosphate and *B. subtilis*, in a descending order. Oxalic acid, monobasic and dibasic sodium phosphate had slight or no effect. Free, conjugated and total phenolic compounds accumulated in roots of treated seedlings with the effective inducers. A positive correlation between the effectiveness of the used inducers and accumulation of phenolic compounds was recognized. Further investigations are needed on this effect as a prophylactic safe measure for crown gall control.

INTRODUCTION

The crown gall bacterium *Agrobacterium tumefaciens* is classified as tumorgenic organism due to its ability to cause neoplastic disease in dicotyledonous plants and economic losses in nurseries (Escobar *et al.* , 2002).

The induced systemic resistance (ISR) has been demonstrated recently as a nonspecific response, active against the crown gall bacterium *A. tumefaciens* (Suo and Leung, 2001), Hence certain biotic and abiotic inducers were considered. Abiotic factors tested included salicylic acid, 2 mM; oxalic acid, 20 mM; dibasic sodium phosphate, 100 mM and monobasic potassium phosphate, 100 mM (Wang Haihua *et al.*, 2002, Atritalla and Brishammar, 2002 and El-Toony, 2003). Among the biotic agents used *Pseudomonas fluorescens* (Singh *et al.* , 2003) and *Bacillus subtilis* (Aly *et al.* , 2002) were considered.

The scientific basis of the induced resistance has emerged with the identification and characterization of biochemical pathways governing resistance in plants against invading pathogen (Shetty *et al.* , 2002). In this regard, Singh *et al* (2003) found that *P. fluorescens* induced the synthesis of phenolics, when it was used as an inducer.

The aim of this study was to investigate the possible use of certain resistance inducers in controlling crown gall disease away from pollution hazards produced by the traditional chemical means.

MATERIALS AND METHODS

Cultures of the causal organism:

Isolation of the causal organism was performed from peach plants grown at Kaha nursery (Kalubia governorate), according to the methods described by Tawfik *et al* (1983). The reference isolate AC12 of *A. tumefaciens* was obtained from the Bacterial Disease Department, Plant Pathology Res. Institute, Giza. Propagation of the bacteria was made in nutrient glucose broth for two days at 28ºC. The inoculum was standardized, turbidimeterically at 560 nm, to give viable count equivalent to $1.6x10^8$. cfu /ml

Bacterial suspension preparation :

B. subtililis isolate No. 108 and *P. fluorescens* 450 were obtained from Bac. Dis. Dpt. , PP. Rresearch Inst. , Giza. Propagation was made on nutrient agar plates for 24 hours at 28ºC. The growth was harvested by adding 20 ml/plate of sterile distilled water and scraping with a wire loop. The turbidity of the bacterial suspension was adjusted to 10^8 cfu per milliliter as determined spectrophotometerically at 560 nm.

Determination of phenolic compounds:

Seedling roots in sprayed and control treatments were collected at 24, 48, 72 and 96 hours after spraying the biotic and abiotic agents. Extraction and determination of free, conjugated and total phenols was made according to the method of Snell and Snell (1953).

Control with biotic or abiotic-agents:

B. subtilis, P. fluorescens as biotic agents and dibasic sodium phosphate, monobasic potassium phosphate, monobasic sodium phosphate oxalic acid and salicylic acid as abiotic factors were used at the concentrations given in Table (1). Leaves of one year old potted Nemaguard peach seedlings were sprayed with 20 ml/seedling of each suspension using a hand glass atomizer. The seedlings of each treatment were divided into four groups, these were sprayed 24, 48, 72 or 96 h. before soil inoculation with 20 ml/pot of pathogenic bacterial suspension $1.6x10^8$cfu/ml.). The disease incidence was evaluated after 30 days from inoculation. Mean number and weight of galls were determined. The gall inhibition percentages was calculated according to the equation:

Gall inhibiton percentage= $100 - [\frac{a}{b} X\ 100]$.

where: a = average weight of galls per seedling for the treatment.

b = average weight of galls per seedling for the control.

The complete randomized design in five replicates was followed. Data were statistically analyzed and tested for confidence at 5% level.

Table 1. Biotic and abiotic-agents against crown gall disease and their concentration.

Inducers		Mol. Weight	concentration
Salicylic acid	($HOC_6H_4CO_2H$)	138. 12	2 mM
Oxalic acid	(HO_2CCO_2H)	90. 04	20 mM
Monobasic potassium phosphate	($KH_2\ PO_4$)	136. 09	100 mM
Monobasic sodium phosphate	(NaH_2PO_4)	119. 98	100 mM
Dibasic sodium phosphate	(Na_2HPO_4)	141. 96	100 mM
Pseudomonas fluorescens			10^8 cfu
Bacillus subtilis			10^8 cfu

RESULTS

Effect of inducers on disease incidence :

Table (2) shows that the tested agents differed considerably in their ability to induce resistance to crown gall disease. Spraying the effective inducers generated a significantly smaller weights and number of galls than spraying with less effective ones. Spraying salicylic acid 72 h before inoculation was the most effective inducer (gall inhibition 94. 9%) followed by *P. fluorescens* (88. 6%), monobasic potassium phosphate (81%) and *B. subtilis* (70. 9%). Oxalic acid, monobasic and dibasic sodium phosphate were least effective and resulted in 5. 1, 3. 8 and zero % gall inhibition, respectively.

There is a possible correlation between the time of application and soil inoculation with the pathogen, on disease incidence. In this respect, seedlings treated 72 h before inoculation developed less disease compared to other treatment.

Table 2. Effect of inducers on gall formation of peach seedlings in relation to the time of inoculation with the crown gall bacterium.

Inducers	*Gall number/seedling				Gall weight/seedling				Gall inhibition %			
	24h	48h	72h	96h	24h	48h	72h.	96h	24h	48h	72h	96h
Salicylic acid	2	2	1	2	1. 7	0. 9	0. 4	0. 8	78. 7	88. 0	94. 9	90. 2
P. fluorescens	3	2	2	3	2. 2	2. 0	0. 9	1. 4	72. 5	73. 3	88. 6	82. 9
KH_2PO_4	3	3	2	3	3. 8	2. 4	1. 5	2. 7	52. 5	68. 0	81. 0	67. 1
B. subtilis	3	3	3	3	4. 0	3. 2	2. 3	3. 6	50. 0	57. 3	70. 9	56. 1
Oxalic acid	5	5	5	5	7. 7	7. 2	7. 5	7. 8	3. 7	4. 0	5. 1	4. 9
NaH_2PO_4	5	4	6	6	7. 9	7. 3	7. 6	8. 1	1. 2	2. 7	3. 8	1. 2
Na_2HPO_4	6	5	6	6	7. 9	7. 3	7. 9	8. 2	1. 2	2. 7	0. 0	0. 0
Control	6	5	6	6	8. 0	7. 5	7. 9	8. 2	0. 0	0. 0	0. 0	0. 0

L. S. D at 0. 05 for gall inhibition
Treatment (T) = 4. 65
Time of application (M)= 3. 29
Interaction TXM= 9. 31.
* Rounded figures.

Determination of phenolic compounds:

Root extracts of peach seedlings were analysed. The relative differences in the amounts of phenols were extrapolated.

Data in Table (3) show the free, conjugated and total phenolic compounds determined as mg catechol/100g fresh weight of root tissues either inoculated or non-inoculated treatments. It is clear that the free and total phenols in the roots of the sprayed seedlings, showed marked increase after spraying with salicylic acid, *P. fluorescens,* monobasic potassium phosphate or *B. subtilis.* Spraying with oxalic acid, monobasic or dibasic sodium phosphate did not induce any significant change in the concentrations of various phenolic groups. Concerning the conjugated phenolic compounds, it is clear that this group followed the same trend of free phenols. The sprayed seedlings, however, contained lower content of such compounds than free phenols. Generally, it could be concluded that free, conjugated and the total phenolic compounds reached the highest concentrations in the root tissues after 72h from spraying compared to other time intervals.

Table 3. Free, conjugated and total phenolic compounds as mg catecol/100g fresh weight of peach seedling root after 24 , 48, 72 and 96 hours form spraying.

Inducers	24 h			48 h			72 h			96 h		
	•	••	•••	•	••	•••	•	••	•••	•	••	•••
Salicylic acid	4. 2	3. 1	7. 3	5. 3	4. 8	10. 1	8. 9	7. 8	16. 7	3. 8	9. 4	13. 2
P. fluorescens	3. 2	2. 8	6. 0	5. 9	3. 1	9. 0	8. 5	5. 6	14. 1	6. 0	4. 3	10. 3
KH_2PO_4	2. 6	1. 9	4. 5	4. 5	1. 7	6. 2	3. 3	6. 9	10. 2	3. 1	4. 9	8. 0
B. subtilis	2. 0	1. 1	3. 1	2. 0	2. 8	4. 8	4. 2	3. 4	7. 6	1. 8	3. 1	4. 9
Oxalic acid	0. 0	0. 3	0. 3	0. 3	0. 2	0. 5	0. 2	0. 3	0. 5	0. 2	0. 3	0. 5
$Na_2H_2PO_4$	0. 2	0. 1	0. 3	0. 1	0. 3	0. 4	0. 3	0. 2	0. 5	0. 3	0. 1	0. 4
Na_2HPO_4	0. 2	0. 0	0. 2	0. 2	0. 2	0. 4	0. 0	0. 3	0. 3	0. 1	0. 4	0. 5
Control	0. 1	0. 1	0. 2	0. 1	0. 3	0. 4	0. 2	0. 1	0. 3	0. 2	0. 2	0. 4

Free phenolic •
Conjugated phenolic ••
Total phenolic •••

L. S. D at 0. 05 for:
Inducers (I) = 1. 27
Time of application (M) = 0. 86
Interaction IXM = 2. 43

DISCUSSION

Many investigators studied the phenomenon of ISR in the context of controlling bacterial diseases (Buonaurio *et al.* , 2002 and Wang Haihua *et al.* , 2002). In the present work, some systemic resistance inducers were tested in an attempt to reduce crown gall disease incidence as an environment-friendly approach. Pretreatment of peach seedlings with such effective inducers (salicylic acid, monobasic potassium phosphate, *P. fluorescens, B. subtilis)* led to resistance to *A. tumefaciens* as indicated by decreasing the disease severity, number and weight of galls developed, thus conforming with results reported by Suo and Leung (2001).

These finding may suggest that spraying the inducers in concern increased non-specific systemic resistance that plays a major role as defence mechanism against *A. tumefaciens* attack. Four different inoculation intervals, i. e. 24, 48, 72 and 96h after treatment with inducers, were tested to roughly determine the onset of resistance taking place. The results indicated that inoculation with *A. tumefaciens* 72 h after spraying showed the least disease incidence and severity.

These results are in harmony with those reported by many investigators working on *A. tumefaciens* oncogenes cause transformed plant cells that over produce auxin and cytokinin (Lee *et al.,* 2003). Two oncogenes encode enzymes that convert tryptophan to indole-3-acetic acid. A third oncogene encode an enzyme which produces cytokinins. The inactivation of the three oncogenes abolishes tumourigenesis. Moreover, the acquired disease resistance can be induced in many plant species against *A. tumefaciens* by a novel synthetic chemical acibenzolar-S-methyl (BTH) as a against *A. tumefaciens* by a novel synthetic chemical acibenzolar-S-methyl (BTH) as a dipping application (Suo and Leung, 2001). Similar responses were reported by Abd-El-Kareem *et al.* , (2001) on the accumulation of chitinase and B-1,3-gluconase in late blight affected leaves. Similar results were obtained by Velazhan *et al.* (1999) on the relationship between antagonistic activities of *Pseudomonas fluorescens* isolates against *R. solani* and their attributes on chitinase and B-1,3-gluconase enzymes. The bacterial metabolites (*B. subtilis* metabolites) also induced systemic resistance against biotrophic pathogens (Stiener and Schonbeck, 1995). *Bacillus subtilis* metabolites induced systemic resistance against biotrophic pathogens by stimulating plants ability to compensate the damaging effects of the pathogens on plant metabolism leading to prolonged maintenance of assimilation rates (Podile and Laxmi, 1998). Our results are in agreement with those of Enebak and Carey, (2000) who found that some plant growth promoting rhizobacteria (PGPR) are able to induce systemic resistance (ISR) in plants against root and foliar diseases. In this regard, Mohamed (1992) reported that phenols were the most pronounced chemical component that could be directly correlated with resistance. In this work, resistance in peach seedlings by the effective used inducers through induction of phenolic compounds was confirmed. These inducers, however, induced the synthesis of phenols with varied levels at different times after spraying. The maximum level of phenols was recorded after 72 h from spraying . Approximately, similar results were also reported by Lux Endrich *et al.*, (2002) and Singh *et al.* , (2003).

This study showed conclusively that the effectively used inducers were able to induce resistance in peach seedlings. Treatment of plants with such inducers may be suggested as an alternative strategy of crop protection with a more satisfactory protection of the environment. Further investigations are needed on the duration of the prophylactic effect of (ISR) in nurseries and established orchards. Also, further studies are recommended to clarify the relationship between time of applying the inducer and the cycle of wound healing, as well as the cell conditioning and transformation and the associated biochemical alterations.

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استحثاث المقاومة الجهازية ضد بكتيريا أجروباكتيريم تيوميفاشينز باستخدام مواد حيوية ومركبات كيماوية

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تحت ظروف الصوبة، تمت تجربة مقاومة مرض التدرن التاجى من خلال المقاومة الجهازية المستحثة بواسطة الاحماض العضوية والاملاح والبكتيريا. حيث تم استخدام كل من حمض الأوكساليك والسالبسيليك، وفوسفات الصوديوم أحادى وثنائى القاعدية وبكتيريا باسلس ساتلس وسيدوموناس فلورسنس كمستحثات، وقيمت هذه المستحثات لاحداث المقاومة الجهازية فى بادرات الخوخ ضد البكتيريا أجروباكتيريم تيوميفاشينز وذلك برش هذه المستحثات على أوراق بادرات الخوخ متبوعاً بعدوى التربة بالمسبب المرضى على فترات من رش المستحث. وأظهرت النتائج أن حمض السالسيلك كان الأكثر تأثيراً فىمقاومة المرض تليه فى التأثير البكتيريا سيدوموناس فلورسنس ثم فوسفات البوتاسيوم أحادى القاعدية فبكتيريا الباسلس ستلس، على الترتيب، بينما كان حمض الأوكساليك وفوسفات الصوديوم أحادية وثنائية القاعدية عديمة التأثير أو ذو تأثير ضعيف جداً وقد اتضح أن المركبات الفينولية الحرة والمرتبطة والكلية قد تراكمت فى جذور البادرات المعاملة بالمستحثات الفعالة حيث وجدت علاقة موجبة بين فعالية المستحثات المستخدمة والكميات المتراكمة من المركبات الفينولية فى الجذور. هذا ويجب مواصلة الدراسة للوصول إلى استمرارية التأثير الوقائى والآمن والقياسى للمقاومة الجهازية المستحثة لمقاومة مرض التدرن التاجى. ودراسة علاقة توقيت رش المتحثات مع دورة التئام الجروح بالأضافة إلى تهيئة وتحويل الخلايا وما يلازمها من تغيرات بيوكيماوية.