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FINAL REPORT

PROJECT NO. I--592-83

**Resistance to Postharvest Pathogens of Tomato
Ripening Mutants and Hybrids Developed for
Extended Storage Life**

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ABSTRACT

A considerable shelf-life extension of fresh tomato fruits has recently been achieved by using non-ripening and slow ripening tomato mutants. However, along with this longer shelf-life, the exposure duration of the fruit to post-harvest pathogens is also exceptionally prolonged. The objectives of the research project were: (1) to evaluate different slow ripening tomato genotypes for resistance to post-harvest pathogens; (2) to study the mechanisms involved in fruit resistance; (3) to improve the understanding of host-pathogen interactions in tomato fruits; (4) to clarify the genetic control of ripening in various aberrant ripening cultivars (Alcobaca, Long Keeper, Garder Peach, Spanish Winter) and genetic stocks (*nor*₂, *nor*₃); (5) to develop adapted inbred and F₁ hybrid varieties with the *nor* and *nor*^A alleles; (6) to compare shelf life and taste panel scores of normal and selected inbred and F₁ hybrid cultivars with the non-ripening (*nor* and *nor*^A) gene; (7) to establish optimum harvest stage and post-harvest treatments required to maximize shelf life and quality of non-ripening (*nor* and *nor*^A) inbreds and F₁ hybrids; (8) to contrast response of mature green fruit from plants heterozygous for the *nor* or *nor*^A allele with normal ripening tomato fruit to Ethephon treatment. Completion of objectives 4-8 is required to establish the commercial feasibility of utilizing these novel germplasm for tomato improvement.

Substantial variation in the degree of fruit ripening inhibition occurs among inbred tomato lines homozygous for the non-ripening (*nor*) gene. This wide variation appears to be a result of more than 2 alleles (*nor* and *nor*^A) at the *nor* locus and modifier genes affecting the expression of these alleles.

Storage and shelf life of nor hybrid fruit is intermediate between the normal and mutant inbreds and varies depending upon maturity stage at harvest and inbred combinations used to produce the hybrid. Harvest of nor hybrid or inbred fruit at early maturity stages frequently results in poor color development in storage; low storage temperatures ($12 \pm 1^{\circ}\text{C}$), result in improved external color versus higher temperature ($20 \pm 1^{\circ}\text{C}$) storage. It appears that some stimulus from vegetative tissues is required during ripening in nor inbred and nor hybrid fruit to encourage fruit color development. This problem is less pronounced in fruit heterozygous for the nor^A allele; these fruit ripen normally and exhibit little shelf life advantage versus normal fruit. Inbred lines of nor^A have been developed which ripen acceptably on the vine and display dramatically improved shelf life versus normal cultivars.

Preliminary shelf life and taste panel studies indicate that long shelf life combined with acceptable flavor can be developed in nor F₁ hybrids as well as nor^A inbreds. Breeding, post-harvest handling, and consumer acceptance studies indicate that this novel germplasm offers substantial opportunity to improve fresh tomato quality and shelf life, however, major changes in handling practices will be required to exploit the advantages of these novel types.

Studies of host resistance to common post-harvest pathogens showed that following penetration into the pericarp, an association exists between the level of the PG activity in the fruit prior to invasion and its resistance to Botrytis cinerea infection. Fungal infection due to Rhizopus stolonifer, B. cinerea and Georticum candidum was found to enhance fruit PG in the normally ripening tomato. In the non-ripening mature fruit, which lacks the ability to synthesize PG, no

inducement of plant PG followed infection and all the PG forms present due to fungal infection were of fungal origin.

Fungal inoculation stimulated ethylene evolution in the pre-climacteric normal fruit and in the non-ripening mutant. A similar pathway of ethylene synthesis was suggested for both fruits.

Exogenic ethylene stimulated spore germination in both the normal and the mutant fruit but enhanced fungal growth indirectly, via stimulation of the ripening process, only in the pre-climacteric normal fruit.

OBJECTIVES OF THE ORIGINAL RESEARCH PROPOSAL

The main objectives of the original proposal were: 1) to evaluate the resistance to post-harvest pathogens of tomato ripening mutants and their hybrids; 2) to study the mechanism of fruit resistance; 3) to use the special host-pathogen system, in which the host is a mutant fruit devoid of parameters of normal ripening, for a better understanding of post-harvest interactions; 4) to continue genetic analyses and post-harvest studies of new fruit ripening variants and 5) to initiate semi-commercial shipping and consumer acceptance studies of adapted inbreds and F₁ hybrids; 6) to assess post-harvest handling requirements of these novel types to optimize shelf life and consumer quality.

I - ISRAEL

A. Evaluation of the tolerance to natural postharvest infection of non-ripening and slow ripening tomato mutants and their hybrids.

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SUMMARY

Fruits of the ripening tomato mutants, nor (non-ripening) and rin (ripening inhibitor) and the slow ripening cultivars Long Keeper and alocobaca, were highly tolerant to natural decay development during storage at 12 and 20°C, as compared with normal ripening fruit. The greatest tolerance was exhibited by the nor mutant. Of the hybrid fruits examined (from crosses of each variant with normal), the nor hybrid was the only one exhibiting relatively high tolerance to infection. Incidence of decay in fruits of this hybrid after 30 days at 12° and 20°C was 50% and 60% of that of normal fruit at 12° and 20°C, respectively. The principal causal decay organisms were Alternaria alternata and Rhizopus stolonifer. The four mutant fruits and the nor - hybrid fruit were markedly resistant to Rhizopus infection during prolonged storage.

INTRODUCTION

Mature tomato fruit is very susceptible to postharvest losses due to decay organisms. Decay development, fruit softening and chilling susceptibility are the limiting factors in storage life of the fresh market fruit.

The tomato ripening mutants nor (non-ripening) and rin (ripening inhibitor) exhibit dramatically prolonged shelf life (Tigchelaar, McGlasson & Buescher, 1978) and greater resistance to storage diseases (Barkai-Golan & Kopeliovitch, 1980; 1981; Robinson & Tomes, 1968). Prolonged shelf-life has also been identified in the slow-ripening cultivars, alcobaca and Long Keeper (Kopeliovitch, Mizrahi, Rabinovitch & Kedar, 1980; Leal & Tabim, 1974). However, their sensitivity to decay has not been yet evaluated.

Fruits of the nor and rin mutants are not suitable for traditional fresh market use since they fail to develop the colour and flavor characteristics of ripened fruit even after exogenic ethylene treatment (Tigchelaar, McGlasson & Buescher, 1978). Utilization of the nor mutant to form hybrids with normal cultivars results in fruits with slower rates of ripening and softening and increased shelf-life whereas less marked effects of rin were found in its hybrids (Buescher, Sistrunk, Tigchelaar & Ng, 1976; Ng & Tigchelaar, 1977; Tigchelaar, McGlasson & Buescher, 1978).

The relative resistance of the nor and rin mutants and their hybrids has already been demonstrated under artificial inoculation conditions (Barkai-Golan & Kopeliovitch, 1981, 1988; Meir-Lavi, 1986; Meir-Lavi, Barkai-Golan & Kopeliovitch, 1988), however, it is important to evaluate the postharvest response of these genotypes under natural storage and prolonged shelf-life conditions. The present

paper also reports on the tolerance to natural pathogens of the slow-ripening cultivars Long Keeper (LK) and alcobaca, which develop some colour while on the plant but ripen abnormally when harvested mature-green (Kopeliovitch, Mizrahi, Rabinovitch & Kedar, 1980; Pressey & Avants, 1982).

METHODS

The following plants were grown in the open field: normal tomato cultivar (n-5-F₈-321-2); its partially isogenic non-ripening nor and rin mutants (n-5-F₈-297-b, and n-5-F₈-297-b, respectively; seventh generation of selves while keeping the nor and rin loci in a heterozygous condition); F₁ nor and rin hybrids (nor/+ and rin/+) the result of a cross between the two; LK (F₈-LKxPU74-32); alcobaca (n-4-F₈-228-2); LK hybrid [LK/+; Floradade x (LK x 82117)]; alcobaca hybrid (alc/+; n-4-F₈-228-2 x n-5-F₈-321-2).

Fruits of the non-ripening mutants nor and rin were harvested one week after the fruit had turned from green to yellow. Fruits of the slow-ripening mutants LK and alcobaca and those of F₁ hybrids and of the normal cultivar were harvested at 'breaker' stage. One hundred fruits of each type were washed in water, dried and stored both at 12°C and at shelf-life conditions at 20°C. The experiment was carried out twice.

The incidence of natural decay and the responsible causal organisms were recorded under storage conditions. Fungal identification followed isolation on potato-dextrose agar (PDA) and incubation at 23°C.

RESULTS AND DISCUSSION

Incidence of decay in storage. Fruits of the four mutants all exhibited high resistance to decay development during storage. The greatest resistance was recorded for the nor mutant, being followed by rin fruit. Incidence of decay in nor and rin fruits reached 4-6% and 6-9% after 30 days of storage, respectively, as compared with 66-71% in the normal fruit, and little further increase in decay was observed after 60 days (Fig. 1 and 3). In LK and alcobaca incidence of decay increased with the prologation of the storage period from 30 to 60 days. This was especially marked during storage at 20°C (Fig. 3).

Incidence of decay of the rin, LK and alcobaca F₁ hybrids was similar to that of normal fruits. After 30 days of storage, decay incidence in these fruits reached 60-64% at 12°C and 69 to 83% at 20°C (Fig. 4).

The nor hybrid was the only combination which exhibited enhanced resistance to postharvest decay as compared to normal fruit, both at cold storage (12°C) and shelf-life conditions (20°C) (Fig. 2 and 4). Incidence of decay in fruits of this hybrid after 30 days of storage decreased by about 50% and 35% of that recorded in the normal fruit at 12° and 20°C, respectively (Fig. 2).

Details of postharvest diseases. In these studies Alternaria alternata (Fr.) Keissler was the most ubiquitous decay organism in all types of fruits. The incidence of Alternaria rot in the normal fruit and in the rin, alcobaca and LK hybrids reached 70-75% of the total decay during cold storage. The rest of the decay was due mainly to Rhizopus stolonifer (Ehrenb ex. Fr.) Lind. During shelf-life, when

the incidence of Rhizopus rot was higher than that during cold storage, the incidence of Alternaria, which is characterized by a much slower rate of growth than that of Rhizopus, was lower than during cold storage, reaching 55-60% of the total. Only very low incidence of Rhizopus rot was found in the stored mutant fruits (mainly in nor, rin and alcobaca) (Fig. 1 and 2). A very low percentage of gray mold, caused by Botrytis cinerea Pers. and of sour rot, caused by Geotrichum candidum Link ex Pers. was recorded in the normal and hybrid genotypes.

The results of this study clearly indicate that fruits of the non-ripening mutants nor and rin are markedly tolerant to decay development under natural infection conditions. This result is in good agreement with the high tolerance of these fruits to R. stolonifer and A. alternata infection as recorded under artificial inoculation conditions (Barkai-Golan & Kopeliovitch, 1981, 1988). In addition, the slow-ripening cultivars, alcobaca and LK as well as the nor hybrid have been shown here to be relatively tolerant to the postharvest organisms. These results suggest that the two slow ripening mutants, which are capable of partial ripening on the plant, and the nor hybrid, which is characterized by marketable colour and flavour, may be of value for fresh market use not only from the point of view of physiological advantages, but also due to their marked lower susceptibility to postharvest diseases.

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Fig. 1. Incidence of decay in nor, rin, alcobaca and Long Keeper fruits, as compared to that in the normal tomato fruit, at cold storage (12°C).

Fig. 2. Incidence of decay in nor, rin, aloabaca and Long Keeper hybrid fruits, as compared to that in the normal tomato fruit, at 12°C.

Fig. 3. Incidence of decay in nor, rin, alcobaca and Long Keeper fruits, as compared to that in normal tomato fruit, at shel-life conditions (20°C).

Fig. 4. Incidence of decay in nor, rin, alcobaca and Long Keeper hybrid fruits, as compared to that in normal tomato fruit, at shelf-life conditions (20°C).

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B. MECHANISMS INVOLVED IN FRUIT RESISTANCE

1. Involvement of fungal penetration in the resistance to infection by Botrytis cinerea of tomato ripening mutants and their hybrids.

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ABSTRACT

Fruits or peel extracts of non-ripening nor and rin tomatoes suppressed germ-tube elongation of Botrytis cinerea spores, as compared with mature normal fruits. Contact inoculation with a diseased tomato fruit was found to be a good indication of the relative resistance to Botrytis infection of the mutant fruits and their hybrids as was exhibited by the prolongation of the incubation period and by the reduced incidence of rotted fruits during shelf-life. The highest level of resistance was exhibited by the nor mutant and its F₁ hybrid, suggesting that this character was transferred from the mutant to the hybrid fruit. Exposure to chilling temperature or hot water treatment prior to

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inoculation markedly increased the sensitivity of normal fruits and partially broke the resistance of the previously resistant fruits. It is suggested that the relative resistance of the non-ripening mutants and the nor hybrid to Botrytis infection is diminished by environmental conditions which favor penetration. The results also suggest the presence of spore germination suppressing factors in the fruits of the mutant tomatoes and their hybrids which may be involved in their resistance to infection.

Storage life of normally ripening varieties is limited by both fruit softening and the development of postharvest pathogens.

Fruits of the non-ripening rin and nor tomato mutants fail to soften and are characterized by a prolonged shelf life (9,10,11). Lacking the normal pigmentation of tomato fruit, these mutants are of limited value in their homozygous state. However, crossing the mutant with a normal ripening cultivar results in F₁ hybrids which produce fruits characterized by both color development and a much reduced rate of softening, as compared with fruits from normal cultivars (4,7). Both mutants and their hybrids were reported to be much less subject to postharvest rots than normal fruits (2,3,5,9).

The present work was aimed at examining the responses of the mutant fruits and their hybrids to infection by Botrytis cinerea Pers., one of the main pathogens of tomato fruit (1,6) and at studying the relationship between fungal penetration into the fruit and its development within the fruit tissues and the relative resistance to infection. Since contact with a decayed fruit serves as a common mechanism for disease initiation in the harvested tomato

fruit (6), studies were carried out mainly under simulated conditions of natural infection.

MATERIALS AND METHODS

The following plants were grown in the open field: normal tomato cultivar (n-5-F8-321-2), its partially isogenic non-ripening nor and rin mutants (n-5-F8-307-b and n-5-F8-297-b, respectively, which are F8 plants with the nor and rin loci in a heterozygous condition) an F₁ nor and rin hybrids (nor/+ and rin/+) - the result of a cross between the two. The fruits were picked at two stages of maturity: 'mature-green' and 'mature'. The mature stage for the non-ripening fruits and their hybrids was defined as about 14 days after their 'turning point' - which is characterized by the changing of peel color from green to yellow or to red - was defined as the 'mature' stage.

For studies of spore germination in fruit peel extracts, peel tissue, 1 mm thick, of the normal and the mutant fruits at two stages of development, was removed and homogenized for 1 min in 95% ethanol (150 ml.50 g⁻¹ f. wt. of tissue) with a Sorval Omnimixer. The filtered residue was washed twice with ethanol, concentrated x 2 in vacuo at 40°C, then partitioned twice with dichloromethane, as described by Prusky et al. (8). The organic material was dried with anhydrous MgSO₄, concentrated in vacuo, and used as peel extracts.

Aliquots of 50 µl of the extracts were applied on 13-diameter millipore disk filters (0.45-µm pore size), which were held on glass slides. Each disk was loaded with 10 µl of an aqueous spore suspension (10⁷ spores/ml) also containing 5% ethanol, 0.05% polysorbate (Tween-20, ICI) and 0.0015% dimethyl sulfoxide.

The spore suspension was prepared from a 7-day-old single spore culture of B. cinerea on PDA. The culture was isolated from postharvest tomato rot.

After 4 h of incubation in a moist chamber, the spores underwent fixation with lactophenol-cotton blue. Percent germination and germ-tube elongation were examined microscopically. Millipore disks without peel extracts, but loaded with aliquots of the spore suspension after ethanol evaporation, serve as controls.

For spore germination within the normal and the nor mutant fruits, millipore disks loaded with 50 μ l of 10^8 /ml spore suspension were inserted into the pericarp and fruits were incubated for 4 h at 18°C.

Inoculation of normal, mutant and hybrid fruits at two stages of maturity was carried out by: (a) inserting into the pericarp, through a 4-mm-deep wound, a uniform-sized inoculum consisting of both mycelium and spores taken from the periphery of a 7-day-old single spore culture on PDA at 18°C; and (b) contact of healthy fruits with diseased mature normal tomato covered with mycelium and conidia.

Radial rate of rot development following inoculum introduction and incidence of infection following the contact inoculation procedure was determined daily for 7 days at 18°C.

Fruit inoculated by the contact procedure were also submitted to chilling and heat treatments by holding it for 3 days at 0°C or for 5 min in hot water at 52°, prior to inoculation.

Five and 30 replicate fruits served for the inserting and the contact inoculation, respectively, and each experiment was repeated twice. The inoculated fruits were held at 18°C and 90-95% r.h.

RESULTS

Spore germination in peel extracts and fruit tissues.
Percent germination in peel extracts of the mature rin and nor fruits did not differ significantly from that recorded in peel extracts of the normal fruit or in the control disks which lacked any peel extract. On the other hand, much lower rates of germ-tube elongation were recorded in the mature nor and the mature-green normal fruit than in the rin or normal fruit at their mature stage. Means of germ-tube elongation in the ripe fruit samples were similar to those in the extract-less control samples (Table 1).

Incubation of conidia within the ripe normal tomato resulted in significantly higher rates of germ-tube elongation than those incubated in the mature-green normal fruit, or in the nor mutant fruit at the two stages of maturity. No significant differences in the percent germination were found in the various fruit types (Table 2).

Table 1 - Percent of spore germination and germ-tube elongation of Botrytis cinerea spores in peel extracts of the normal and mutant tomato fruits^a

Source of fruit peel extract	Percent germination	Germ-tube length (μm)
Mature-green normal	76.5±3.1 _c	32.6±3.5 ^c
Mature normal	80.3±2.8	49.9±4.7
Mature <u>rin</u>	78.4±2.1	40.4±4.1
Mature <u>nor</u>	74.5±4.4	28.9±2.2
Control ^b	78.2±2.1	48.5±2.2

^a After 4 h at 18°C.

^b Spore germination in the absence of extracts.

^c Standard error of the mean.

Table 2 - Percent spore germination and germ-tube elongation of Botrytis cinerea spores in the normal and nor tomato fruits at two stages of maturity^a.

Fruit type	Percent germination	Germ-tube length (μm)
Mature-green normal	84.9±4.7 ^b	18.4±0.5
Mature normal	89.3±3.1	36.1±2.1
Mature-green <u>nor</u>	83.8±5.2	18.2±1.0
Mature <u>nor</u>	85.7±6.6	19.7±1.1

^a After 4 h at 18°C.

^b Standard error of the mean.

Fungal development in the normal and in the mutant fruits and their hybrids. Following inoculation into the pericarp, rate of B. cinerea development was found to be slower in the nor mutant fruit than in the rin fruit and the normal fruits at both stages of maturity. This was expressed by the prolongation of the incubation period of the disease and the decreased rot diameter during the holding period. For each fruit, rot diameter increased with maturity 6 (Fig. 1).

Fig. 1 - Growth rate of Botrytis cinerea in rin and nor mutants and their hybrids following inoculation into the pericarp.

Following contact inoculation with a diseased tomato fruit, the nor mutant and its hybrid were both markedly more resistant to Botrytis infection than the normal and the rin fruits, the effect being more pronounced at the mature-green stage, when no infection occurred during 5 and 3 days, respectively. In addition to the prolongation of the incubation period of the disease, the relative resistance of the nor mutant and its hybrid was also expressed by the markedly reduced incidence of decay during shelf-life (Table 3).

Table 3 - Incidence of *Botrytis cinerea* decay in the normal tomato fruit, and in rin and nor mutants and their hybrids (rin/+ and nor/+) at 18°C after contact inoculation with a diseased fruit or following exposure to extreme temperatures prior to the contact inoculation.

Stage of maturity and fruit	Incidence of decay (%) ^a after					
	-----2 days-----			-----7 days-----		
	A	B	C	A	B	C
Mature-green						
normal	5.2±1.1b	8.6±1.1	9.9±1.2	54.6±1.3	64.7±1.6	70.9±1.3
<u>rin</u>	4.6±1.3	5.9±2.2	9.8±1.6	49.8±2.1	64.3±1.3	69.3±1.4
<u>nor</u>	0	0	0	5.4±1.3	49.1±1.7	59.4±1.3
<u>rin</u> /+	4.9±1.9	5.6±1.3	8.6±1.4	53.8±1.4	66.2±1.6	69.7±1.2
<u>nor</u> /+	0	0	5.9±1.3	14.9±1.3	49.6±1.4	59.6±1.3
Mature						
normal	14.8±1.3	14.9±1.6	19.8±1.3	80.0±2.1	90.2±2.4	100
<u>rin</u>	9.8±1.1	9.9±1.6	19.7±1.2	75.0±2.4	84.6±3.7	95.0±2.1
<u>nor</u>	0	0	7.7±1.4	14.7±2.0	65.0±2.2	68.7±2.4
<u>rin</u> /+	9.3±1.9	9.6±2.1	19.8±1.2	73.8±1.2	85.5±1.2	94.8±2.3
<u>nor</u> /+	0	0	9.9±1.6	24.6±1.3	69.8±2.1	74.3±2.1

- ^a Each value is the average of 30 replicates.
- ^b Standard error of the mean.
- ^c After contact inoculation.
- ^d After exposure to 0°C for 3 days prior to contact inoculation.
- ^e After exposure to hot water at 52° for 5 min prior to contact inoculation.

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Incidence of decay increased markedly in all the contact-inoculated fruits following their exposure to chilling temperature or to hot water treatment prior to inoculation (Table 3). Although the extreme temperature pre-treatments resulted in increased sensitivity to decay of the nor mutant and its hybrids, their relative resistance as compared with the normal fruit, as well as with the rin mutant and hybrid, was still retained. This was exhibited by both the prolongation of the incubation period and the reduction in the incidence of decayed fruits. Furthermore, whereas in the normal fruit the disease spread all over the fruit by the end of the holding period, at 18°C, in the nor mutant and its hybrid the lesion was more restricted, leaving the rest of the fruit firm. A similar restricted decay was recorded in the rin mutant and its hybrid, although incidence of infection in these fruits was similar to that in the normal fruit (not shown).

DISCUSSION

Extracts of the mutant fruits were found to suppress spore germination of B. cinerea, germ-tube elongation being a more sensitive stage than initiation of germination. The inhibitory effects on germ-tube elongation were confirmed in vivo, when spores were allowed to germinate within fruit tissues of the nor mutant at both the mature-green and the mature stage. The rate of germ-tube elongation was similar for nor mutant mature-green and mature fruits as well as normal mature-green fruits. This result represents half the rate of germ-tube elongation in normal ripe fruits.

The prolongation of the incubation period detected in the nor mutant following inoculation of B. cinerea into the pericarp of the fruit at both stages of maturity is probably

the result of the inhibitory effect of the mutant tissues to the initial stages of hyphal growth. However, once the incubation period had terminated, rate of fungal development in the mutant fruits was similar to that in the normal fruit. On the other hand, contact inoculation with a diseased tomato fruit covered with mycelium and spores was found to be a good means for indicating the relative resistance of the nor mutant fruit and its hybrid to B. cinerea infection, as compared with all the other genotypes tested. The highest level of resistance was exhibited by the nor mutant. The pronounced resistance of the nor hybrid, as compared not only with the normal fruit but also with the rin mutant or its hybrid, suggests that this character was transferred, to a large extent, from the nor mutant to its F_1 hybrid.

The resistance of the nor mutant and nor hybrid to Botrytis infection under a close contact with plenty of fungal inoculum suggests an association with the response of the periderm of these fruits to fungal penetration. This suggestion was further supported by the results obtained from exposing the fruits to extreme temperatures prior to inoculation. Applying either chilling or high temperature treatment partially broke the resistance of the nor fruit or its hybrid to Botrytis infection, thus resulting in increased incidence of decay during shelf-life conditions. Chilling temperatures are well known to predispose the cold-sensitive tomato fruit to B. cinerea infection (6) and were previously found to markedly increase the susceptibility of the nor mutant to Rhizopus stolonifer infection (2). Hot water treatment, which results in removal of the natural wax layer from various parts of the tomato surface (12), seems to increase the sensitivity of the fruit to infection by preparing suitable sites for fungal

penetration. The ability of the two pre-inoculation treatments to decrease the differences in sensitivity between the normal and mutant fruit may support the assumption that the relative resistance of the mutant fruit and its hybrid is associated, to a large extent, with the ability of the pathogen to penetrate the fruit. When this obstacle is removed, fungal development is inevitable, although some fungal suppression in the resistant fruit seems to be directly related to the effect of its peel and pericarp tissues on initial stages of hyphal growth.

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3. Relationship between the pectolytic and cellulolytic activity of Botrytis cinerea Pers. and the resistance to infection of non-ripening tomato mutants and their hybrids.

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Abstract

Enzymes of Botrytis cinerea were detected in vitro using various carbon sources. Pectin-pectate as a sole carbon source induced both polygalacturonase (PG) and pectin lyase (PL) activity, whereas carboxymethylcellulose served as an inducer for C activity. PG activity appeared earlier than C activity when induced by their respective sources. Both PG and PL activities were detected earlier and their level was higher on cell walls of the normal tomato fruit than of the nor mutant, and in each case activity was higher on cell walls of the mature fruits than of the mature-green ones. Whereas relatively high rates of PG and PL activity were recorded on autoclaved tomato homogenate (TH) of both the normal and the nor fruits, only trace levels of PG activity were recorded on unautoclaved media, except for those prepared from ripe normal fruits, and no PL activity was detected on either of the unsterilized media.

Botrytis - infection resulted in PG activity in the enzyme-less rin and nor mutant fruits at both stages of

maturity and in the normal and hybrid fruits at their mature-green stage. In the ripe normal and hybrid fruits, infection increased the level of PG activity recorded prior to inoculation. An association was drawn between the low PG activity recorded in the nor mutant and its hybrid at initial stages of invasion and their resistance to infection.

Following infection an increase in the level of C activity over that recorded in healthy fruits was found in all the tomato genotypes, whereas no PL was recorded in either healthy or infected fruits.

Introduction

The two non-ripening rin and nor tomato mutants develop fruits which fail to soften and are devoid of pectolytic enzymes during all stages of development, except for trace levels recorded in the mature nor fruit (Buescher and Tigchelaar 1975, Ng and Tigchelaar 1977, Barkai-Golan and Kopeliovitch 1983, 1986). Both rin and nor fruits exhibit C-cellulase activity which increases with maturation (Buescher and Tigchelaar 1977, Barkai-Golan and Kopeliovitch, 1983). However, the level of C activity recorded in the nor mutant is markedly lower than that recorded in the normal fruit or its rin mutant (Barkai-Golan and Kopeliovitch 1983). A lower polygalacturonase (PG) activity was recorded in rin and nor hybrids as compared to that in the normal fruits, with the rin and nor hybrids as compared to that in the normal fruits, with the rin heterozygote having the higher activity of the two hybrids (Tigchelaar et al. 1978).

The mutant and the hybrid fruits were both reported to be more resistant to postharvest pathogens than the normal

tomato fruit (Barkai-Golan and Kopeliovitch 1981, 1986, Buescher and Tigchelaar 1977, Robinson and Tomes 1968). Recently, the relative resistance of these fruits to infection by Rhizopus stolonifer (Ehrenb. ex Fr.) Lind and Botrytis cinerea Pers. has been attributed, to a large extent, to the inability of the fungi to penetrate into the pericarp (Barkai-Golan and Kopeliovitch 1981, Meir-Lavy 1986). However, relative resistance to B. cinerea infection of the nor mutant fruits was also found following both fungal introduction into the pericarp and when B. cinerea was allowed to penetrate the fruit directly through floral organs, as was expressed by the prolonged incubation period and by the more restricted mycelial development at the stem-end area, respectively (Meir-Lavy 1986). These phenomena suggested the involvement in fruit resistance of additional factors, probably associated with the medium that each fruit presents to fungal growth and pathogenicity.

Botrytis cinerea is known to produce PG, pectin-lyase (PL) and C, the activity being highly influenced by cultural conditions and by host tissues (Hancock et al. 1964, Brown and Adikaram 1982, 1983, Verhoeff and Warren 1972). Wasfy et al. (1978) and Di Lenna et al. (1981) suggested that virulence of different isolates of B. cinerea was correlated with their ability to produce considerable quantities of PG.

The present work was aimed at studying the pectolytic and cellulolytic potential of B. cinerea in vitro and within the tissues of the normal tomato fruits, its non-ripening rin and nor mutants and their hybrids, and to determine the relationship between enzymatic ability and fruit resistance to infection.

Materials and Methods

Fungal growth in vitro

Botrytis cinerea Pers. was isolated from postharvest decay of normal tomato fruits, cv. 'Faculta 38', and a 7-day old single spore culture on PDA at 18°C was used to prepare a spore suspension. Aliquots of 1 ml of 10^6 /ml spore suspension were used to inoculate 40 ml of a mineral medium prepared according to Winstead and McCombs (1961). The following carbon sources were added to the mineral medium: 0.5% sodium polyepectate (NaPP, Sigma) with 0.5% citrus pectin grade I (Sigma), 1% carboxymethylcellulose (CMC, Sigma), and 1% glucose. Combinations of 0.5% glucose with either NaPP or CMC at 0.5% were also used. When a glucose solution was used, it was autoclaved separately before being added aseptically to the autoclaved minimal medium.

Dehydrated cell walls were prepared from the pericarp of the normal cultivar 'Faculta 38', the non-ripening rin (n-5-F₂297-b) and nor (n-5-F₂307-b) mutants and their hybrids, according to Cooper et al. (1981). The fruits were picked at two stages of maturity: 'mature-green' and 'mature'. For the non-ripening fruits, 14 days after their 'turning point' - which is characterized by the changing of peel colour from green into yellow - was defined as the 'mature' stage. Cell wall preparations were dried at 60°C for 24 h, ground to a homogenous powder, and stored aseptically in a dessicator. Cell wall powder was added to the minimal medium at 1% as a sole carbon source.

Tomato homogenate (TH) medium was prepared by blending for 2 min normal or mutant fruits at two stages of maturity (pH 4-4.3).

The cell wall and TH media, either autoclaved or non-autoclaved, were inoculated with aliquots of B. cinerea spore suspension, as described above.

Inoculated media were held for 5-10 days in a shaking bath at 18°C.

Fruit inoculation

The harvested fruit, picked at two stages of maturity, were inoculated by introducing into the pericarp at 4 mm depth a uniform sized disk consisting of both mycelium and spores taken from the periphery of a 7-day old single spore culture on PDA at 18°C. Non-inoculated fruit served as control. The fruits were incubated for 7 days at 18°C.

Extraction and enzyme assay

Extracts for enzyme assay were obtained from inoculated liquid media after various periods of incubation by filtration through Whatman No. 1 filter disks. Extraction from inoculated TH was done by centrifugation at 12,000 rpm for 10 min at 4°C using the supernatant liquid for enzymatic activity assay.

Extracts from infected fruit were prepared by homogenizing fruit samples, including rot area with a 1 cm diameter of fruit tissue around it, in three volumes (w/v) of 1 M NaCl at pH 9, for 2 min in an Osterizer blender followed by filtration of the liquid fraction, as described previously (Barkai-Golan and Kopeliovitch, 1983). The extracts were centrifuged at 12,000 rpm for 10 min at 4°C to obtain a supernatant for enzyme activity assay.

PG, PL and C activity in the in vitro and in vivo extracts was determined viscometrically. The reaction

mixture consisted of 5 ml enzyme preparation with 10 ml of 1% NaPP (Sigma) in 0.05 M citrate buffer (pH 4.8) for determination of PG activity, 0.9% citrus pectin grade I (Sigma) in 0.1 M Tris-HCl buffer (pH 8.0) containing 0.002 M CaCl_2 - for PL activity or 1% CMC (Sigma) in 0.1 M acetate buffer (pH 5.0) - for C activity. Relative enzyme activity was expressed as percentage loss of viscosity after 60 min incubation in a 200 Volac viscometer at 30°C.

Enzyme assays in vitro and those in inoculated fruit samples were accompanied by determination of mycelial dry weight and rot diameter, respectively.

Results

Effect of substrate source on enzyme activity

The highest level of PG and PL activity of B. cinerea was recorded in the presence of pectin-pectate as the carbon source. Activity in the inducive medium was already been detached 24 h after inoculation and increased gradually until the fifth and seventh day of incubation, respectively (Fig. 1). In the presence of glucose as the sole carbon source, only a low pectolytic activity was recorded whereas the level of mycelial dry weight was highest under these conditions. The addition of glucose to the inducive pectin-pectate medium markedly decreased the level of both PG and PL activity recorded prior to glucose application, while the pattern of activity during fungal development was retained (Fig. 1).

C activity was highest in the presence of CMC as the sole carbon source, whereas only low PG and PL activity was recorded in the CMC medium. Fungal growth in the CMC medium was similar to that in the pectin-pectate medium. The lowest

C activity was found in the glucose-containing medium. The addition of glucose to the inducive CMC medium resulted in a marked decrease in the level of C activity recorded prior to glucose application.

The activity of PG was detected earlier than that of C when induced by their respective sources (Fig. 1).

The pectolytic activity of B. cinerea on cell walls - containing medium strongly depended on the origin of the cell walls. Both PG and PL activity was detected earlier and its level was higher on cell walls delivered from the normal fruit than from the nor mutant. In each case, a higher activity was found on cell walls of the mature stage than of the mature-green stage. Mycelial dry weight was higher on cell walls of normal than of nor fruits at both stages of maturity (Fig. 2).

Fungal PG and PL activity on autoclaved TH prepared from either the normal or the nor fruits at both stages of maturity, has already been detected 24 h after inoculation. Enzymatic activity increased with fungal development until the seventh day after inoculation whereas fungal dry weight continued to rise during the entire holding period (Table 1).

On autoclaved TH made from mature-green normal fruits or from both mature-green and mature nor fruits, only very low PG activity was detected following B. cinerea inoculation. No PL was recorded on unautoclaved TH prepared from either the normal or the nor mutant at both stages of maturity (Fig. 3).

Fig. 1. Effect of carbon source on polygalacturonase (PG), pectin lyase (PL) and cellulase (C_x) activity, of *Botrytis cinerea* in relation to its mycelial growth. ○ - pectin-pectate; ● - pectin-pectate + glucose; □ - CMC; ■ - CMC + glucose; ▲ - glucose.

Fig. 2. Polygalacturonase (PG) and pectin lyase (PL) activity of *Botrytis cinerea* and fungal growth on cell wall media originated from normal tomato and its non-ripening nor mutant fruit at their mature-green (o) and mature (●) stages.

Fig. 3. Polygalacturonase (PG) and pectin lyase (PL) activity of *Botrytis cinerea* on sterilize (S) and unsterilize (US) tomato homogenate of a normal tomato and its non-ripening nor mutant fruit at their mature-green (MG) and mature (M) stages.

Fungal growth on the autoclaved TH was characterized by a heavy mass of spore bearing mycelium. On the anautoclaved media, excluding that prepared from ripe tomato fruit, growth was very sparse, as was expressed by the low values of mecelial dry weight during the entire holding period (Table 1).

Table 1 - *Botrytis cinerea* growth on sterilized (S) and unsterilized (US) tomato homogenate (TH) of a normal and nor mutant fruits at their mature-green (MG) and mature (M) stage.

Origin of TH (Fruit)	Dry Weight (mg) after (days) ^a			
	2	4	6	10
<u>Normal</u>				
S - MG	52	148	480	520
US - MG	0	12	15	19
S - M	58	169	495	590
US - M	42	158	438	542
<u>nor</u>				
S - MG	47	138	440	530
US - MG	0	11	15	18
S - M	49	143	490	540
US - M	0	9	16	20

^a At 18°C.

Fig. 4. Polygalacturonase (PG) and cellulase (C_x) activity in normal, nor and rin tomato fruits following inoculation with Botrytis cinerea.

Enzymatic activity in vivo

Botrytis cinerea infection resulted in PG activity in fruits of rin and nor mutants at both stages of maturity and in fruits of the normal and hybrid fruits at their mature-green stage - all of which were devoid of activity when healthy. Activity was detected 24-48 h after inoculation and increased with disease development (Figs. 4 and 5). The level of PG activity in the two mutants, and mainly in the nor mutant, was markedly lower than in the normal fruit and in each fruit type activity increased with maturity.

Fungal infection resulted in an increase in the cellulolytic activity normally found in healthy fruits of the normal and the mutant genotypes. This increase was only evidenced 3 days after inoculation when the disease was already in a progressive stage (Fig. 4).

In the ripe stage of the normal or the hybrid fruits, fungal infection also increased the level of PG activity found prior to infection, the effect being detected 24 h after inoculation (Fig. 5).

Fig. 5. Polygalacturonase activity in normal tomato fruit and its two hybrids nor/+ and rin/+ following inoculation with Botrytis cinerea.

Discussion

The production of PG by B. cinerea was found to precede that of C when induced in vitro by their respective inducers. However, pectin-pectate containing medium, which induced PG and PL production, was also suitable for C production, whereas CMC-medium, an inducer of C-cellulase, caused a repression of both PG and PL during fungal growth.

The presence of glucose as a sole carbon source or its addition to either of the inducers markedly repressed synthesis of both the pectolytic and cellulolytic enzymes. The glucose effect is a common phenomenon for various microorganisms (Patil and Dimond 1968, Spalding et al. 1973), and a selective repression by glucose of either C or PG has also been reported (Horton and Keen 1966, Lisker et al. 1975).

The earlier appearance of PG than C activity was also observed in vivo following Botrytis inoculation of the normal, the mutant and the hybrid fruits. The sequence of enzyme activity may be of importance in fungal pathogenesis since the early production of a cell wall degrading enzyme at the infection site may be responsible for the initial stages of invasion and fungal establishment (Horton and Keen 1966). Wasfy et al (1978), working with different isolates of B. cinerea, correlated fungal virulence with its ability to produce PG whereas no correlation was drawn between virulence and PL production (Di Lenna et al. 1981).

Although B. cinerea introduction into the pericarp resulted in PG activity in fruits of all the genotypes, a markedly lower level of activity at the initial stages of invasion was typical for the nor mutant and its hybrid as compared with the normal fruit. This suggests an association

between the pectolytic activity of the pathogen within the fruits, and their resistance to infection.

A correlation was found between the level of the pectolytic activity of a tomato genotype prior to inoculation and the enzymatic ability of the pathogen within its tissue. In vitro studies show that the pectolytic activity of B. cinerea is induced by soluble pectic compounds in the medium. In a normal tomato fruit, where the middle lamella is hydrolyzed during ripening by fruit enzymes (Hobson and Davies 1971), an increase in the water soluble compounds may serve as inducers for pectolytic activity of the pathogen. In the non-ripening tomato mutants, which are devoid of pectolytic enzymes, such an inducement seems to be lacking.

The inability of B. cinerea to produce PL within any of the fruits or on unautoclaved TH, in spite of its potential activity on inductive synthetic medium or on autoclaved TH of the different genotypes, may suggest the presence of cell wall proteins in the tomato tissues which inhibit PL activity. Denaturation of the proteins by autoclavization would then prevent inhibition and result in PL activity. Recently, cell wall proteins extracted from normal tomato fruits were found to inhibit both PG and PL produced by Glomerella cingulata, but only PL produced by B. cinerea (Brown and Adilaram 1983). The inhibition of PL activity in B. cinerea-infected tomato fruits was found to be common to both the normal and the mutant fruits, indicating a lack of association between PL activity and fruit resistance.

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4. EFFECT OF PEEL INJURY AND ENZYMATIC ACTIVITY OF THE FRUIT
ON THE TOLERANCE OF TOMATO GENOTYPES TO ALTERNARIA
INFECTION

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Abstract

Differences in the tolerance to Alternaria alternata infection between various tomato genotypes were found to be greatly related to their response to wounding or to factors favouring fruit injury.

Heat treatments, which resulted in a partial removal of the natural wax of tomato, as well as exposure to chilling conditions, markedly increased the susceptibility of the non-ripening nor mutant to infection. It was suggested that the ability of the pathogen to penetrate into the pericarp has a primary role in the relative resistance of tomato genotypes to Alternaria infection.

Following penetration into the pericarp a higher rate of lesion development was recorded in the mature normal fruit than in the mature mutant or hybrid fruits. However, rate of growth in the latters was higher than in the mature-green normal fruit. A correlation was drawn between the cellulolytic activity of the pathogen within the fruit

tissues and the level of enzymatic activity of the fruit prior to infection.

1. Introduction

Alternaria alternata (Fr.) Keissler is the main postharvest pathogen of tomato fruit, comprising about 80% of the total rot incurred during storage (Barkai-Golan, 1973).

In contrast to the normally ripening fruit, the non-ripening nor and rin mutants, as well as the nor hybrid were found to be markedly less subject to postharvest Alternaria rot under natural infection conditions, both during storage at 12°C and under shelf-life conditions (Barkai-Golan and Kopeliovitch, 1980, 1986).

The fungus is known as a weak pathogen, penetrating the fruit through wounded and injured tissue (Barkai-Golan, 1973; Pearson and Hall, 1975; Ramsey et al. 1955; Toledano, 1985). Similarly, exposure of normal tomato fruits to extreme temperatures was found to predispose them to Alternaria invasion (McColloch and Worthington, 1952; Toledano, 1985).

The present study reports on the rate of development of A. alternaria lesions in rin and nor mutants and their hybrids as compared with the normal ripening fruit following inoculation of the fruit pericarp. The relative resistance to infection of the normal and mutant fruits was also studied after exposure to high temperature or chilling conditions prior to inoculation.

Another approach to evaluate the resistance of the various tomato genotypes to infection was to study the enzymatic activity of the pathogen within the fruit tissues. The present study reports on the relation between the relative resistance to infection of the different tomato genotypes and their relative ability to act as substrates and inducers of pathogen cellulolytic and pectolytic activity.

2. Materials and methods

Rate of fungal development in inoculated fruits.

Rin and nor mutants as well as the normal cultivar were grown in glasshouses at 22°C, and harvested at their mature-green and mature stage. The fruits were surface sterilized with 70% alcohol and then inoculated by inserting a 3mm³ disk of mycelium and spores into the pericarp through a 5-mm-deep wound. Colony disks were taken from the periphery of a 10-day old single spore culture of A. alternata on PDA at 23°C. Rate of lesion development was measured during 15 days at 23°C in nine replicate tests.

The effect of wounding, chilling conditions and high temperature on disease development.

Normal and nor fruits, harvested at two stages of maturity and surface sterilized with 70% alcohol, underwent either of the following treatments prior to inoculation:

- (a) pricking with 4 mm needles at four sites on the fruit;
- (b) exposure to 0°C for three days; (c) immersion in a hot water bath at 52°C for 3 min. Inoculation was done by immersing the fruit in a 10⁸/ml of A. alternata spore suspension.

Spores were collected from a 10-day old single spore culture of A. alternata as described above. Uninjured fruits dipped in the same spore suspension served as a control. Twenty replicate fruits were used per each stage of maturity and each treatment.

Enzymatic activity in inoculated fruits

Normal, nor and nor hybrid (nor/+) fruits at two stages of maturity were inoculated by introducing into the pericarp a uniform sized disk taken from an A. alternaria colony on PDA, as described above. Non inoculated fruit served as the control. The enzymatic activity in fruit extracts was examined during 10 days at 23°C.

Samples from infected fruit consisted of rotted tissue along with a 1 cm section of the adjoining unrotted area.

Extracts were prepared by homogenizing these samples in three volumes (w/v) of 1 M NaCl at pH 8, for 2 min in an Osterizer blender. The homogenate was held at 4°C for 40 min, centrifuged for 10 min at 12,000 rpm at -2°C and filtered, as described previously (Barkai-Golan and Kopeliovitch, 1983).

Polygalacturonase (PG) and Cx-cellulase activity in the supernatant was determined viscometrically. The reaction mixture consisted either of 5 ml enzyme preparation with 10 ml of 1% sodium polypectate (NaPP; Sigma) in citrate buffer (pH 4.8) for determination of PG activity or 1% carboxymethyl cellulose (CMC; Sigma) in acetate buffer (pH 5.0) for Cx activity. Relative enzyme activity was expressed

as percentage loss of viscosity after 60 min incubation in 200 Fenske Ostwald viscometers at 30°C.

3. Results

Disease development in the different genotypes

Following artificial inoculation into the pericarp, rate of rot development was markedly higher in the mature fruits than in the mature-green ones. No marked differences in rot development were found between the mutant, hybrid and normal fruits at the latter stage of maturity. At the mature stage, however, the highest rate of growth was found in the normal fruits. After 15 days of storage at 23°C rot diameter on these fruits reached 55 mm, as compared to only 40 mm on the nor and rin mutants. Rate of fungal development in the two mature hybrids was slower than that in the mature normal fruit but faster than in the corresponding mutant fruits (Fig. 1 and 2).

The effect of wounding, chilling temperature and hot water treatment on the incidence of infection.

Wounding the fruit prior to inoculation resulted in a similar low incidence of decay (12-15%) in the normal and the mutant fruits. However, marked differences were recorded between the two fruit types at their mature stage. Whereas inoculation of the normal fruits resulted in 72% infection, only about 35% infection was recorded for the inoculated nor fruits (Fig. 3).

Submitting the fruits to a chilling temperature or hot water treatment greatly increased their susceptibility to A. alternata infection as compared with the untreated fruits

and lessened the difference in resistance to infection between the mutant and the normal fruits. However, differences in response to infection between the normal and the mutant fruits were still exhibited by the longer incubation period recorded in the mature-green mutant fruits than in the corresponding normal fruits (Fig. 3).

In both the normal and the nor fruits incidence of decay following exposure to extreme temperatures was higher than that following artificial pricking (Fig. 3).

Enzymatic activity in the normal, mutant and hybrid fruits.

Fungal infection resulted in pectolytic activity in the enzyme-less nor mutant at both stages of maturity and increased the low activity recorded in the mature-green normal and hybrid fruits. The level of PG activity in the normal fruit was markedly higher than in the mutant or hybrid fruit.

In the mature normal and hybrid fruits A. alternata only slightly increased the level of PG activity found prior to infection (Fig. 4).

Following infection, a marked increase in Cx activity was recorded in the three genotypes at both stages of maturity. This increase was evidenced 2 days after inoculation in the normal fruit and 3 days after inoculation in the mutant and hybrid fruits (Fig. 5). In general, the levels of Cx activity in the normal fruit were markedly higher than those in the nor and nor/+ fruits at each stage of maturity. Furthermore, the levels recorded in the mutant and hybrid fruits at their mature stage were similar to those found in the normal cultivar at its mature-green stage (Fig. 5).

Fig. 1. Rate of lesion development in *Alternaria*-inoculated *nor* tomato fruits and their hybrids.

Fig. 2. Rate of lesion development in *Alternaria*-inoculated *rin* tomato fruits and their hybrids.

Fig. 3. The effect of wounding (B), chilling conditions (C) and hot water dip (D) on the incidence of decay in Alternaria-inoculated normal and nor fruits, as compared with untreated fruits (A).

Fig. 4. - Polygalacturonase activity in normal, nor and nor-hybrid tomato fruits following inoculation with Alternaria alternata.

Fig. 5. Cellulase activity in normal, nor and nor-hybrid tomato fruits following inoculation with Alternaria alternata.

Discussion

Similar to the results obtained with fruit under natural infection conditions (Barkai-Golan and Kopeliovitch, 1980, 1986), the non-ripening tomato mutants were markedly more resistant to Alternaria infection than the normal cultivar also following artificial inoculation.

The effect of wounding on the increase in the incidence of decay in the nor and rin fruits suggests that the ability of the pathogen to penetrate into the pericarp of these genotypes has an important role in their resistance to Alternaria infection. The fact that pre-inoculation exposure of the fruit to chilling injury or hot water treatment markedly reduces or eliminates the differences in response to infection between the normal and the mutant fruit strengthens this assumption. The involvement of fungal penetration through the peel in the resistance of the fruit to infection is in good agreement with the results obtained with Rhizopus stolonifer - infected tomatoes (Barkai-Golan and Kopeliovitch, 1981). However, whereas a similar rate of Rhizopus growth was recorded in both the normal and the mutant fruits following inoculation into the pericarp differences in the rate of Alternaria growth were found in the different tomato genotypes.

Examination of the enzymatic activity of A. alternata in inoculated fruits showed that PG activity was markedly lower than Cx activity in all the fruit types. It was shown

that when none or a low level of fruit PG activity was recorded in the healthy tomato fruit, fungal infection then resulted in the increase of this level. However, in the mature normal or hybrid fruits, with a high PG activity prior to inoculation, fungal infection did not contribute to a further increase in the PG activity. On the other hand, a correlation was found between the level of the cellulolytic activity of a tomato genotype prior to inoculation and the enzymatic activity of the pathogen within its tissues. This correlation is in good agreement with the importance related to the cellulolytic activity of A. alternata in its pathogenicity to tomato fruits (Toledano, 1985). The present findings lead to the suggestion that while the ability of the pathogen to penetrate into the pericarp has a primary role in the relative resistance of the mutant and hybrid fruit to Alternaria infection, the cellulolytic ability of the pathogen which succeeded in penetrating the fruit will affect its establishment and development.

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Polygalacturonase activity and origin in the normal, nor mutant, and nor hybrid tomato fruits following Botrytis cinerea infection.

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Mature-green fruit from normally ripening lines of tomato (Lycopersicon esculentum) and from its nonripening nor mutant line, both of which are devoid of pectolytic enzymes (5, 6), exhibited polygalacturonase (PG) activity following Rhizopus stolonifer infection (1). The enzymes produced under these conditions were found to be of fungal origin (2). However, in the mature normal tomato fruit, which is characterized by the production of plant PG, Rhizopus infection resulted not only in the accumulation of fungal enzymes but also in the enhancement of tomato PG (2). In the mature nor fruit, which contains no PG, except for trace levels that have sometimes been recorded (5), no enhancement of plant enzyme followed fungal infection (2). There is no information on the ability of other postharvest pathogens to induce tomato PG during pathogenesis or on the difference in reaction to infection between the normally ripening fruit and the enzyme - less nor mutant.

The present study reports on the effects of the pectolytic fungus Botrytis cinerea, which is an important

postharvest pathogen of tomato fruit, capable of infecting different tomato genotypes (4), on the pectolytic activity of the ripening fruit and the enzyme-free nor mutant. It also reports the effects of both B. cinerea and R. stolonifer on the enzymatic activity of the nor/+ fruit, which is characterized by a limited PG accumulation (3).

Botrytis cinerea produced PG activity when grown on autoclaved tomato homogenate (TH) and multiple forms of PG were detected on polyacrylamide gels.

No trace PG enzyme activity was found in the uninfected mature-green fruit of the normal tomato cv, its partially isogenic non ripening nor mutant and F1 nor hybrid (nor/+) as well as in the mature nor fruit. The level of enzyme activity in the uninfected hybrid fruit was 27% of that in its mature normally ripening counterpart.

Following infection by B. cinerea, little PG activity was detected in the mature-green normal fruit but over a 3-fold increase in activity was recorded for the mature normal fruits over that for the uninfected fruits. Botrytis infection only marginally increased the enzymatic activity in the mature-green and mature nor fruit and the mature-green nor/+ fruit. The PG activity of the mature hybrid fruit was not increased by B. cinerea infection, although this activity was markedly increased in both the mature-green and mature hybrid fruit following R. stolonifer infection.

Using a rocket immunoelectrophoresis procedure neither the TH medium nor the Botrytis-inoculated medium showed any immunoreactive material after immunoelectrophoresis with antiserum to tomato PG, indicating that the pectolytic

enzymes of the fungus did not cross-react with tomato PG-specific serum. This analysis also showed that the mature-green normal, nor and nor/+ fruits did not contain tomato PG whether infected or not.

In the mature normal fruit B. cinerea highly increased the amount of tomato PG protein. In the mature nor fruit no tomato PG was detected prior to or after fungal infection. In the uninfected mature nor/+ fruit a lower amount of tomato PG was found by this method, and this amount was not increased by either Botrytis or Rhizopus infection.

In a previous study we have shown that R. stolonifer infection results in an enhancement of tomato PG production and activity in the normal ripe tomato but not in the non-ripening mutant fruit (2). The present study suggests that the ability of fungal infection to induce tomato PG might be a general phenomenon in the normally ripening fruit which is a typical PG producer but not in the mutant fruit where PG would otherwise not be expressed.

In our nor/+ fruit, which is characterized by limited PG formation comprised mainly of PG1, no enhancement of tomato PG was induced by either R. stolonifer or B. cinerea infection suggesting that PG acceleration is exhibited when higher levels of PG, including PG1 and PG2, exist in the fruit tissues.

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3. Stimulation of fruit ethylene production by wounding and by Botrytis cinerea and Geotrichum candidum infection in normal and non ripening tomatoes.

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Abstract

Inoculations with both Botrytis cinerea and Geotrichum candidum stimulated ethylene evolution in the pre-climacteric normal tomato fruit and the non-ripening nor mutant which did not show any rise in ethylene when uninfected. In the post-climacteric normal fruits, new peaks in ethylene production were formed. The rise in ethylene evolution in all types of infected fruits has already been detected during the incubation period of the disease. Ethylene peaks were detected earlier and were higher in fruits infected with B. cinerea than with G. candidum, coinciding with the faster rate of growth of the former. Mechanical wounding also stimulated ethylene synthesis by the non-ripening fruits, production being directly proportional to wound dimension. Considerably higher rates of ethylene were recorded for infected fruits than for

of ethylene were recorded for infected fruits than for mechanically-injured fruits in which wound dimensions were similar to those of lesion development.

Applying AOA at the site of inoculation inhibited ethylene production by 55-60% in the normal fruits and by about 80% - in the nor mutant fruits. A similar pathway of ethylene synthesis was suggested for normally ripening tomato fruit and non-ripening infected tissues.

Fruits of the non-ripening nor and rin tomato mutants, which are devoid of respiratory climacteric and ethylene evolution characteristic of normal tomato fruit (Tigchelaar et al. 1978) exhibited climacteric-like respiration and ethylene evolution in response to infection by Rhizopus stolonifer (Ehrenb. ex. Fr.) Lind (Barkai-Golan and Kopeliovitch 1983). It was suggested that some factor associated with fungal infection stimulates the synthesis of ethylene by the non-ripening mutants. This factor may be similar to that which stimulates ethylene synthesis in wounded rin mutant fruits (Herner and Sink 1973) or in pericarp disks cut from rin and nor fruits (McGlasson et al. 1975). However, no information is available on the part which 'wound ethylene' may have in the total level of ethylene produced by infected fruit tissues, as well as on the ability of other fruit pathogens to stimulate mutant fruit tissues to synthesize ethylene.

The present study reports on the response of the normal tomato fruit and the nor mutant to Botrytis cinerea Pers. and Geotrichum candidum Link. two common post-harvest

pathogens of tomato fruit (Barkai-Golan 1981), and evaluates the contribution that mechanical injury may have in the total level of ethylene produced during pathogenesis.

The origin of ethylene produced by infected tissues of the normal and the mutant fruit was also studied, using aminooxyacetic acid (AOA), a potent inhibitor of ethylene of plant origin (Yang 1985).

Materials and Methods

Plant materials

The normal tomato cultivar 'Faculta 38' and the nor mutant, (n-5-F8307-b) were grown in a heated glasshouse. Fruits were harvested at two stages of maturity 'mature-green' and 'mature'. For both the normal and the mutant fruits, the stage when the fruits have attained full size but are still green is referred to as the 'mature-green' stage. For the normal fruit, the stage when more than 90% of the surface has turned red is referred to as the 'mature' stage, while, for the nor mutant, 14 days after their 'turning point' - which is characterized by the turning of peel colour from green to yellow - is referred to as the 'mature' stage.

Fungal cultures and inoculations

Botrytis cinerea and Geotrichum candidum were isolated from a diseased normal tomato fruit. Spore suspensions were prepared from a 7-day-old single spore culture grown on potato dextrose agar (PDA) at 18°C.

Inoculations of the fruits were made by injecting 25 μ l of 10^6 /ml spore suspension of each of the fungi into the pericarp of freshly harvested fruit (each weighing about 100 g) as described previously (Barkai-Golan et al. 1987). Some of the fruit were treated with aminooxyacetic acid (AOA)(sigma), by pre-injection of 20 μ l of 10 mM of AOA solution, at the B. cinerea inoculation site 30 min prior to inoculation. Pricked uninoculated fruits, or fruits into which aliquots of deionized water were introduced, served as controls. The experiments consisted of five replicates.

Ethylene determinations

Fruits were placed individually inside 0.5 l glass flasks which were sealed with rubber serum caps for 1 h at 18°C before a gas sample was withdrawn with a hypodermic syringe. Ethylene content was determined daily over a period of 9 days following inoculation. In parallel with the ethylene determinations, the rate of disease development at 18°C was also recorded.

Ethylene evolution was also measured from mature-green and mature normal and nor tomato fruits wounded by cutting a "window" of variable size (5mm², 10mm², 20mm² or 40mm²) in the pericarp. The fruits were placed individually in sealed glass flasks as described above and ethylene content was determined 6 h after wounding. Six fruits were used for each wound dimension and ripening stage. Ethylene evolution was also measured from tissue disks (4mm²) cut from the centre rotted area of the lesion 5 days after inoculation with B. cinerea.

Ethylene evolution by B. cinerea grown in vitro on PDA or on tomato homogenate (TH) was measured daily during 9 days in a shaking bath at 18°C. TH media were prepared from tissues of normal and mutant fruits at the two stages of maturity as described previously (Barkai-Golan et al. 1987). Aliquots of 0.5 ml of 10⁶ spore suspension served to inoculate 25 ml of the medium in 50 ml Erlenmeyer flasks. The flasks were sealed for 1 h before a gas sample was withdrawn.

Ethylene production was measured with a gas chromatograph (Packard).

Results

B. cinerea and G. candidum infection markedly stimulated ethylene evolution from fruits of the non-ripening nor mutant at both the mature-green and the mature stages resulting in an evolution pattern typical of the normal climacteric tomato fruit (Figs. 1 and 2). The rise in ethylene evolution from infected nor fruits was already detected 24 h after inoculation with both fungi, before the termination of the incubation period of the disease in these fruits. Maximal values of ethylene from infected nor fruits were detected 4 and 8 days after inoculation with B. cinerea and G. candidum, respectively, with much higher peaks being recorded for the Botrytis - infected fruits. The time of peak ethylene evolution in the infected fruits was the same for both stages of maturity for each of the fungi.

While no difference in the peak level of ethylene evolution was found between mature-green and mature

Geotrichum - infected nor fruits ($12.5 \mu\text{l kg}^{-1} \text{h}^{-1}$), the peak level of ethylene recorded in mature-green Botrytis - infected nor fruits was higher than in their mature counterparts (29 and $21 \mu\text{l kg}^{-1} \text{h}^{-1}$, respectively).

In the pre-climacteric mature-green normal fruits, fungal infection advanced the initiation of ethylene evolution. As in the nor fruits, the rise in ethylene evolution was detected one day after inoculation with each of the pathogens. However, a maximal level of $47 \mu\text{l kg}^{-1} \text{h}^{-1}$ of ethylene was recorded on the fourth day after inoculation with B. cinerea whereas a peak of $12 \mu\text{l kg}^{-1} \text{h}^{-1}$ was recorded on the eighth day after inoculation with G. candidum.

In the ripe normal fruits, picked at the post-climacteric stage, both B. cinerea and G. candidum resulted in the formation of new climacteric peaks with rates higher than those of the uninfected fruits. However, maximum ethylene evolution was obtained on the third and sixth day after Botrytis and Geotrichum inoculation, respectively, and reached levels of 48 and $18 \mu\text{l kg}^{-1} \text{h}^{-1}$.

Of the two fungi studied, B. cinerea exhibited a faster rate of growth when fruits were inoculated. For each of the fungi, the shortest incubation period and most rapid rate of growth occurred in the mature normal fruit (Figs. 1 and 2).

Applying AOA prior to inoculation markedly inhibited ethylene evolution from B. cinerea - infected fruits. Maximal levels of ethylene production by the normal tomato fruit were reduced by 63% and 54% at its mature-green and mature stage, respectively, whereas a reduction of about 80%

Fig. 1. Ethylene evolution from normal ('Faculta 38'), and nor mutant tomato fruits, at their mature-green (A) and mature (B) stages of maturity ($\bullet$), following inoculation with Botrytis cinerea ($\blacktriangle$), or following AOA treatment prior to inoculation ($\blacksquare$). A relationship is drawn to fungal development (o--o).

Fig. 2. Ethylene evolution from normal ('Faculta 38') and nor mutant tomato fruits, at their mature-green (A) and mature (B) stages of maturity (●) and following inoculation with Geotrichum candidum (▲). A relationship is drawn to fungal development (o--o).

was recorded for the nor fruits at both stages of maturity (Fig. 1).

No ethylene was evolved from disks taken from the centre of areas of B. cinerea on normal or mutant fruits, as well as from B. cinerea grown in vitro on PDA or on TH prepared from either normal or the mutant fruit (data not shown).

Mechanical wounding of the normal and the mutant fruit resulted in the stimulation of ethylene production at both their mature-green and mature stage of development. In each fruit type, the level of ethylene increased with the increase in wound dimensions from 5mm³ to 40mm³. Higher levels of ethylene were induced in the mature-green normal fruit than in the nor fruit at either stage of development. Only traces of ethylene were recorded for the nor fruits or the mature-green normal fruits prior to wounding (Table 1).

Table 1

Effect of wound dimension on ethylene production by normal and nor tomatoes.

Wound dimension ^a	Ethylene production ($\mu\text{l kg}^{-1} \text{ h}^{-1}$) ^b			
	normal		<u>nor</u>	
	mature green ^c	mature ^d	mature green ^c	mature ^{d,e}
A	0.4	6.6	0.3	0.3
B	2.0	8.4	1.5	1.7
C	2.8	9.3	2.2	1.7
D	3.9	10.8	2.5	2.2
E	11.0	11.6	5.0	3.5

a A - Unwounded fruit. B - 5 mm³, C - 10 mm³, D - 20mm³, E - 40 mm³.

b After 6 h at 18°C.

c Mature-green - fruits harvested and wounded when full size but still green.

d Mature normal - fruits harvested and wounded when more than 90% of the surface has turned red.

e Mature nor - fruits harvested and wounded 14 days after they have turned from green to yellow.

Discussion

Both B. cinerea and G. Candidum stimulated ethylene evolution from the non-ripening nor fruit which lacks an active ripening system and does not response to exogenic ethylene treatment (Tigchelaar et al. 1978). This result, being in good agreement with the effects of R. stolonifer on the nor and rin mutants (Barkai-Golan and Kopeliovitch 1983) suggests the existence of a general phenomenon of host-pathogen interaction involving non-ripening host fruits and soft rot fungal pathogens.

Of the two fungi assayed, B. cinerea induced higher rates of ethylene production than G. candidum when normal or nor fruits were inoculated. This coincides with the faster rate of growth recorded for B. cinerea in all inoculated fruits. A correlation between the level of ethylene production and the rate of lesion development was recorded in citrus fruits inoculated with various pathogens (Schiffmann-Nadel 1974). On the other hand, markedly higher rates of ethylene were evolved from both B. cinerea and G. candidum - infected tomatoes than from R. stolonifer - infected fruits, although R. stolonifer is characterized by a shorter incubation period and a considerable faster rate of lesion development in both the normal and the mutant fruit (Barkai-Golan and Kopeliovitch 1983). The lower rates of ethylene produced in Rhizopus - infected fruits may be the result of the rapid maceration and disorganization of infected tissues, leading to the death of cells and their inability to synthesize ethylene (Ayres 1984).

Alterations in ethylene synthesis by the non-ripening fruits were also induced by mechanical injury. This result is in agreement with the known ability of an injury to stimulate ethylene synthesis by some unripe fruit tissues although the intact fruits at the same stage of development produce no significant amount of ethylene (Yang and Pratt 1978). However, whereas a climacteric-like pattern of evolution was induced in infected fruits, ethylene evolution from the wounded tissue continued to rise with the increase in wound dimensions until more than half of the fruit was removed.

Comparison of rates of ethylene produced by wounds of certain dimensions with those produced by fungal lesions of similar sizes, indicated that considerably higher values were recorded in the latter case. Thus, a peak of 21-29 $\mu\text{l kg h}^{-1}$ ethylene was recorded in Botrytis - infected nor fruits on the fourth day after inoculation, and a peak of about 12 μl was recorded in Geotrichum - infected nor fruits on the eighth day after inoculation. A mechanical wound of dimensions similar to those caused by lesion development (20 and 40 mm^2 , respectively) resulted in the production of only 2.2-5.0 $\mu\text{l ethylene kg}^{-1} \text{ h}^{-1}$. These results may suggest that, in addition to the mechanical stress produced during pathogenesis, another factor is involved in the synthesis of infection ethylene. This biological or 'parasitic factor' seems to differ for different fungi and is dependent on host tissue. The lack of agreement between wound-ethylene and infection-ethylene is also demonstrated by the low levels of post-peak infection-ethylene produced despite the increased dimensions of the lesion formed at that time.

The inhibitory effect of AOA on ethylene production in B. cinerea - infected fruits was clearly shown. Since AOA is known as an ACC synthase inhibitor, and thus acts as a specific inhibitor of ethylene biosynthesis by higher plants (Boller et al. 1979), the above mentioned results suggest that ethylene production in both the normal and the non-ripening mutant fruit is of plant origin being induced by the pathogen. The inability of an abundance of fungal elements in the centre of the rot or under cultural conditions to produce ethylene supports this suggestion. These results are in agreement with the considerable amount of ethylene produced by healthy, symptomless tissue at the margin of R. stolonifer rot, where only rare penetration of fungal hyphae was noted (Barkai-Golan and Kopeliovitch 1983).

The ability of the nor mutant to produce ethylene as a reaction to fungal infection and its inhibition by AOA application suggest a similar pathway of ethylene synthesis by the normally ripening fruit and the non-ripening infected tissues. The inability of AOA to arrest totally the production of infection-ethylene, seems to result from the short lag time between its application and fungal inoculation, thus preventing a sufficient uptake by the tissue.

The pathway for synthesis of wound ethylene is believed to be similar or identical to the pathway for synthesis of non-wound ethylene in ripening fruit tissue and is similarly inhibited by amino-vinylglycine (AVG; Yang and Pratt 1978). Inhibition of ethylene production by AOA during pathogenesis may therefore consist of the inhibition of wound ethylene induced by lesion progress and that induced by the extra 'parasitic factor' typical of each pathogen.

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DESCRIPTION OF COOPERATION

During the period of the present grant, exchange visits were made. Prof. E.C. Tigchelaar of Purdue University visited the Volcani Center, Bet Dagan and The Faculty of Agriculture, Rehovot, in 1984 at the early stages of the research. During that time Prof. Tigchelaar gave a seminar on tomato ripening mutants, discussions were held and detailed plans were prepared. During August 1985 Prof. R. Barkai-Golan of the Volcani Center visited Purdue University where she gave a seminar on the results of the first year's studies. A short visit by Dr. E. Kopeliovitch, of the Faculty of Agriculture, in Purdue at the same time enabled discussion among the three collaborators of the present project. During her partial sabbatical leave in 1986, Prof. Barkai-Golan spent about two months at Purdue University where she joined Prof. Tigchelaar in his studies on the tolerance to postharvest decay of the American tomato cultivars.

EVALUATION OF THE RESEARCH ACHIEVEMENTS WITH RESPECT TO THE AIMS OF THE ORIGINAL RESEARCH PROPOSAL AND THE BENEFIT TO AGRICULTURE.

The present research progressed according to the major aims of the project as planned.

The relative resistance to postharvest pathogens of various tomato ripening mutants and their hybrids was evaluated both under cold storage conditions (12 °C) and during shelf-life. As a result of this evaluation a breeding programme has been carried out in both the U.S.A. and in Israel.

The variation in the ripening of different nor lines appears to be due to the presence of more than 2 alleles at the nor locus as well as modifier genes affecting their expression. Storage and shelf life of nor hybrid fruit is intermediate between the normal and mutant inbreds and varies depending upon maturity stage at harvest and the inbred combinations used to produce the hybrid. Inbred lines of nor^A have been developed which

ripen acceptably on the vine and display dramatically improved shelf life versus normal cultivars.

Preliminary shelf life and taste panel studies indicate that long shelf life combined with acceptable flavor can be developed in nor F₁ hybrids as well as nor^A inbreds. Breeding, postharvest handling and consumer acceptance studies indicated that this germplasm offers substantial opportunity to improve fresh tomato quality and shelf life.

Evaluation of the resistance to postharvest infection under natural infection conditions and after artificial inoculation procedures, as well as the better understanding of the mode of penetration of the pathogen into the fruit greatly contributed to the improvement of the storage life of fresh tomatoes. This was achieved by combining the criteria of slow physiological senescence with host tolerance to infection.

The use of several host-pathogen systems consisting of a mutant fruit devoid of normal pectolytic activity as well as ethylene evolution and a pectolytic fungal pathogen led us to a better understanding of several new aspects of postharvest host-pathogen interactions. These included the determination of the relative contribution of the host and the pathogen to the PG produced in normal and mutant tomatoes during infection with several storage pathogens and of the origin of the ethylene in infected fruits. Host-pathogen studies also contributed to the understanding of the effects of exogenic ethylene on disease development in the ripening and non-ripening fruits and served as a basis for studies of polyamine biosynthesis in infected tomato fruits and its interaction with ethylene.

LIST OF PUBLICATIONS

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6. Lavy-Meir, Gila, Barkai-Golan, Rivka and Kopeliovitch, E. (1988). Initiation at the flowering stage of postharvest Botrytis stem-end rot in normal and non-ripening tomato fruits. Ann. Appl. Biol. 112: 393-396.
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