# Resistance of Healed Citrus Exocarp to Penetration by *Penicillium digitatum*

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## ABSTRACT

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Germination of *Penicillium digitatum* was delayed on healed citrus exocarp. Hyphae were unable to penetrate healed tissue and were confined within cell walls or collapsed cells at the injured surface. Contents of these hyphae were electron dense and the cell walls were distorted. In contrast, the hyphae that successfully penetrated unhealed tissue contained normal organelle constituents and cell walls. During healing of injured cells, phenolic substances and ligninlike materials were identified with various histological stains at 16 and 30 hr after injury. The inability of hyphae to penetrate healed tissue could not be attributed to lack of sufficient nutrients or water for growth or to increased physical strength of the lignified, healed tissue. Resistant tissue that had healed for 30 hr was as easily macerated as fresh tissue by exopolygalacturonase produced by *P. digitatum*, but tissue healed for 72 hr was less susceptible to such maceration. Release of reducing groups from healed tissue upon maceration was less than from freshly injured exocarp. The accumulation of phenolic and ligninlike materials in cell walls of injured exocarp cells appeared to be the major factor involved in resistance of healed tissue to penetration by *P. digitatum*. These materials may inhibit fungal growth, interfere with enzymatic degradation and/or lignify penetrating hyphae.

*Additional key words*: *Citrus sinensis*, lignification, postharvest diseases, wound healing.

Infection of citrus fruits by *Penicillium digitatum* Sacc., the cause of green mold, is dependent upon injuries to the rind for release of nutrients to stimulate spore germination and for entry of invading hyphae. Injuries involving only the exocarp, however, possess the capacity to heal and may become resistant to penetration by *P. digitatum*, if high relative humidities (RH) and temperatures are maintained following injury (3). Under such conditions, phenylalanine ammonia-lyase is activated (12) and phenolic compounds (11), ligninlike materials (5), and lignin (5,11,12) accumulate. Resistance of healed injured citrus exocarp to penetration by *P. digitatum* has been associated with this accumulation, and we have assumed that these materials either inhibit growth of the fungus or form a physical barrier to penetration. In previous studies, lignin identification in healed citrus exocarp was determined with the Wiesner test (phloroglucinol-HCl) (5,11,12). However, specificity of this test for lignin has been questioned (23).

The purpose of this study was to explore the role of nutrition, moisture, enzymatic degradation, and physical strength in the resistance of healed exocarp to penetration by *P. digitatum*. Additional staining procedures were utilized to ascertain the identity of lignin.

## MATERIALS AND METHODS

Mature, naturally colored fruit of *Citrus sinensis* (L.) Osbeck 'Valencia' were washed and injured abrasively in a single area with coarse 0.423-mm (60-grit) sandpaper (11) and quickly immersed in running tap water to remove phytotoxic peel oil. Injured fruit were immediately held at 30 C and near 100% RH for 24 hr to induce healing, or at ambient RH (30–50%) to prevent it (3). In some experiments, fruit with fresh injuries were used for unhealed controls. Injuries were inoculated with spores of *P. digitatum* ($2 \times 10^5$/ml) suspended in distilled water or nutrient solutions (24) containing Triton X-100 as a surfactant. Each treatment was applied to five replicated lots, each containing 12 fruit. The fruits were stored at 26 C near 100% RH.

Observations of growth on and penetration into unhealed and healed citrus exocarp were made by preparing tissue as previously described for transmission (4) and scanning (2) electron microscopy. Histochemical tests for phenolics and lignins were done with either fresh or fixed injured exocarp. Fresh material was sectioned without embedding at a thickness of 25–30 μm. Tissue fixed in 3% glutaraldehyde, dehydrated with increasing concentrations of tertiary butyl alcohol to 100%, and embedded in paraffin was sectioned at 10 μm. Tests for phenolics utilized ferric chloride-potassium ferricyanide ($FeCl_3$-$K_3Fe(CN)_6$) (14,18) and diazonium salt formed with Fast Red Salt B reagent (20). Lignin was detected by staining with phloroglucinol-HCl (13), chlorine-sulfite (21), toluidine blue (17), crystal violet-erythrosin B (8), and safranin-fast green (8). Responses of injured exocarp to these various stains were observed at 16 and 30 hr after injury and in some instances after 3 and 5 days.

Physical strength of healed and unhealed exocarp was measured as newtons ($kg/m/sec^2$) required to puncture the exocarp (25 fruit per treatment) with a 0.64-cm-diameter flat-tipped metal rod mounted in an Instron model 1122 universal testing instrument (Instron Corporation, Canton, MA 02021) according to the procedure described by Churchill et al (7). Water potential of healed and unhealed exocarp (three disks per treatment) was determined by removing a disk (10-mm diameter and 2 mm thick) and inserting it into a micro-chamber to measure equilibrium RH with a thermocouple psychrometer (Wescor Inc., Logan, UT 84321) (6).

Exopolygalacturonase (exo-PG) produced by *P. digitatum* was prepared (1) from decayed citrus fruit tissue to evaluate degradation of healed and unhealed exocarp. Pieces of exocarp (10 per treatment, 2 mm wide × 5 mm long × 1 mm thick) were placed into the enzyme solution, pH 4.5, and incubated at 23 C for 16 hr. Cohesiveness was measured by the method of Mount et al (16). Release of reducing groups (15) from healed and unhealed exocarp was determined after 4 hr at pH 4.5 and 23 C. Data were adjusted for reducing groups released from tissue not treated with enzyme.

## RESULTS

*P. digitatum* grew rapidly on injured, unhealed exocarp. Abundant hyphae were observed on the surface 24 hr after inoculation (Fig. 1A). Hyphae invading unhealed exocarp

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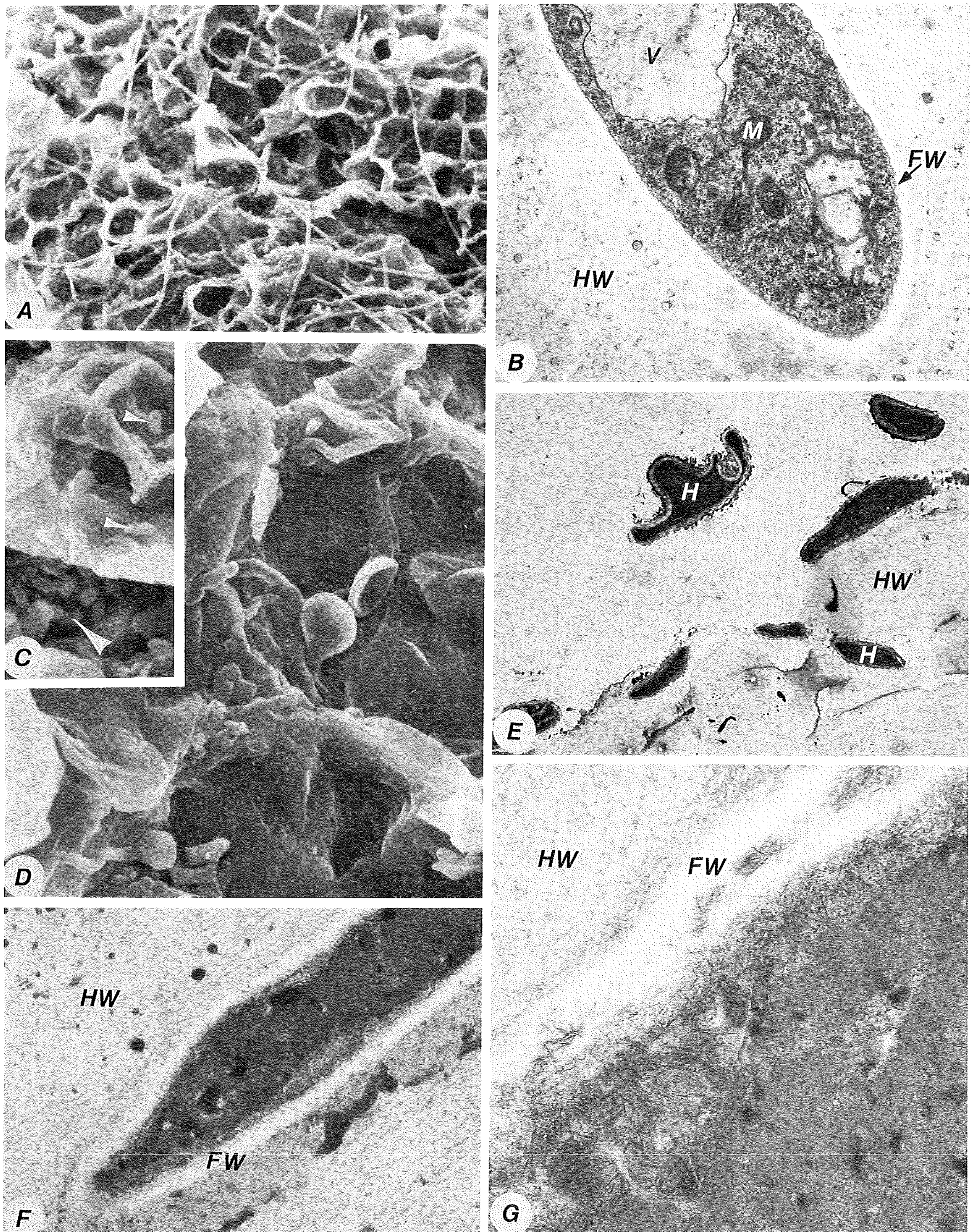


**Fig. 1.** Hyphae of *Penicillium digitatum* growing on and in unhealed and healed exocarp of cultivar Valencia orange. **A,** Hyphal growth on the surface of unhealed exocarp 24 hr after inoculation (×530). **B,** Normal morphology of a hypha during penetration of unhealed exocarp 24 hr after inoculation (×19,250). **C,** Ungerminated spores (arrows) on the surface of healed exocarp 24 hr after inoculation (×680). **D,** Germinating spores on the surface of healed exocarp 48 hr after inoculation (×1,625). **E,** Hyphae on and within cells of healed exocarp 66 hr after inoculation (×4,600). **F,** Electron-dense hypha within a collapsed cell at the healed exocarp surface 66 hr after inoculation (×49,500). **G,** Fibrillar material associated with cell wall and dense protoplasm of deteriorated hypha (×63,450). FW = fungal cell wall, H = hypha, HW = host cell wall, M = mitochondrion, V = vacuole.

appeared normal with the usual organelle components and the typical electron-transparent fungal cell wall (Fig. 1B). Penetration by hyphae of *P. digitatum* was similar, whether unhealed tissue was formed by a fresh injury or by storage of injured fruit for 24 hr at ambient RH. In contrast, growth on healed tissue was slower as evidenced by ungerminated spores scattered on the surface 24 hr after inoculation (Fig. 1C). By 48 hr, however, healed surfaces contained numerous germinating spores (Fig. 1D). At 66 hr after inoculation, hyphae on healed exocarp exhibiting resistance to *P. digitatum* were restricted to the injury surface or within adjacent collapsed cells and their cell walls (Fig. 1E). Contents of the hyphae were quite dense (Fig. 1E and F), and the protoplasm often had collapsed into the cell lumen away from the hyphal cell wall (Fig. 1F). The area between the hyphal cell wall and protoplasm and portions of the wall contained electron-dense fibrillar material (Fig. 1G).

Host cell walls and the contents of collapsed cells in a layer one to two cells deep at the injured surface of healed exocarp were stained by the various stains used to detect phenolics and lignin. Staining was usually most intense in portions of the wall nearest the exocarp surface. Positive reactions for phenolic compounds ($FeCl_3$–$K_3Fe(CN)_6$ and Fast Red B Salt) were obtained in healed tissue removed from the exocarp 16 hr after injury (Table 1). Lignin was detected in healed tissue with toluidine blue at 16 hr after injury, and at 30 hr with phloroglucinol-HCl and chlorine-sulfite. Lignin was not discerned with safranin-fast green or crystal violet-erythrosin B in healed exocarp until at least 3 days after injury. More accumulation of lignin was evident by 5 days. All lignin stains were positive for naturally occurring vascular lignin of the exocarp. Phenolics and lignins were not detected in unhealed tissue.

Nutrients in the form of potato-dextrose broth or orange juice media added to spores of *P. digitatum* did not increase susceptibility to decay of fruits with healed injuries (Table 2). Fruits with fresh, unhealed injuries developed significantly more green mold than did those with healed injuries following inoculation.

The physical forces required to puncture healed and unhealed tissue (Table 3) differed quite substantially. Unhealed exocarp tissue after dehydration at low RH was much more resistant to penetration than was healed tissue. Healed injuries were slightly more difficult to penetrate as healing progressed than were freshly made injuries (Table 3).

Moisture levels within injured exocarp of fruits exposed to RH favorable or unfavorable for healing are shown in Table 4. Water content of injuries decreased during the initial 24 hr of healing at high RH, then increased until the content after 96 hr at high RH was near the moisture level that was present immediately after injury. Moisture levels within injuries of fruits held at low RH to prevent healing for the first 24 hr after injury were lower (−68.0 bars). However, when fruits were placed under high RH at inoculation, moisture contents increased because of absorption from the surrounding moist atmosphere. Water content of unhealed exocarp at 48 or 96 hr after injury still did not increase to levels that were observed with healed tissue at comparable times.

With healing, injuries exhibited some resistance to cell wall degradation by exo-PG (Table 5). Less reducing groups were released from tissue healed for 30 hr than from fresh tissue, and even lower amounts were obtained from injured tissue healed 48 or 72 hr. At 30 hr, no difference was observed in the strength of healed and fresh tissue (Table 5) after treating with exo-PG. However, tissue healed for 48 or 72 hr was more difficult to macerate than was tissue healed 30 hr or fresh tissue.

TABLE 1. Comparison of different histological tests for the determination of phenolics and lignin in healed exocarp of cultivar Valencia oranges at 16, 30, and 72 hr following injury

| | | Time (hr) after injury | | |
|---|---|---|---|---|
| Stain | Test | 16[a] | 30 | 72 |
| $FeCl_3$–$K_3Fe(CN)_6$ | Phenolics | + | + | |
| Fast Red B Salt | Phenolics | + | + | |
| Phloroglucinol-HCl | Lignin | − | + | |
| Chlorine-Sulfite | Lignin | − | + | |
| Toluidine Blue | Lignin | + | + | |
| Safranin-Fast Green | Lignin | − | − | + |
| Crystal Violet-Erythrosin B | Lignin | − | − | + |

[a]+ = positive, − = negative response.

TABLE 2. Green mold of injured cultivar Valencia oranges inoculated with spores of *Penicillium digitatum* suspended in water or nutrients

| | Nutrients[a] | | |
|---|---|---|---|
| Type of injury | Water | PDB | OJM |
| Fresh[b] | 48.3[c] | 50.0 | 50.0 |
| Healed[d] | 11.7 | 25.0 | 11.7 |

[a]PDB = Difco potato-dextrose broth; OJM = orange juice medium.
[b]Fruit were inoculated immediately after injury.
[c]Values are based on the average number of decayed fruit in five replications each with 12 fruit. Fruit were stored at 26 C near 100% relative humidity.
[d]Fruit were injured, held 24 hr at 30 C and 100% relative humidity, and then inoculated. Values within a column are significantly different, LSD ($P = 0.05$) = 19.5.

TABLE 3. Force required to puncture healed and unhealed exocarp of cultivar Valencia oranges

| Injury | Force (newtons)[a] |
|---|---|
| Uninjured | 56.8 ± 2.3[b] |
| Fresh | 53.2 ± 1.3 |
| Healed, after 30 hr at 100% RH | 64.2 ± 1.6 |
| Healed, after 48 hr at 100% RH | 76.2 ± 1.7 |
| Unhealed, after 30 hr at 30–50% RH | 117.8 ± 4.8 |

[a]$kg/m/sec^2$.
[b]Data are the mean and standard error.

TABLE 4. Water potential of healed and unhealed injured exocarp of cultivar Valencia oranges at various times after injury

| Injury | Healing time (hr) | Water potential (−bars) |
|---|---|---|
| Fresh | | 10.8 ± 0.1[a] |
| Healed[b] | 24 | 30.3 ± 1.8 |
| | 48 | 15.5 ± 0.6 |
| | 96 | 13.8 ± 0.9 |
| Unhealed[c] | 24 | 68.0 ± 1.2 |
| | 48 | 38.7 ± 2.3 |
| | 96 | 24.9 ± 1.2 |

[a]Data are the mean and standard error.
[b]Healed at 30 C and 100% relative humidity.
[c]Unhealed at 30 C and 30–50% relative humidity.

TABLE 5. Release of reducing groups and maceration of freshly injured and healed exocarp of cultivar Valencia after exposure to exopolygalacturonase produced by *Penicillium digitatum*

| Injury | Reducing groups[a] ($\mu g$) | Maceration rating[b] |
|---|---|---|
| Fresh | 21.6 | 1 |
| Healed[c], 30 hr | 18.0 | 1 |
| Healed, 48 hr | 16.9 | 2 |
| Healed, 72 hr | 16.9 | 3 |

[a]Recovered from 50 mg tissue.
[b]Rating based on evaluation of 10 pieces of tissue per treatment: 0 = least resistant, 5 = most resistant.
[c]Healed at 30 C and 100% relative humidity.

## DISCUSSION

Ride (19) suggested five ways by which lignin might hinder fungal invasion of plant tissue. Lignification may make walls more resistant to mechanical penetration, render cell walls resistant to dissolution by fungal enzymes, restrict diffusion of enzymes and toxins from the fungus to the host and, conversely, water and nutrients from the host to the fungus. In addition, phenolic precursors of lignin and free radicals produced during polymerization may inactivate the fungus. Finally, hyphae may be lignified.

In this study, germination of spores of *P. digitatum* on healed exocarp was delayed, but resistance was manifested by the restriction of hyphae to the healed surface or within adjacent cells. The cells within healed citrus exocarp accumulated phenolics and lignins within 30 hr of injury. The identity of lignin as determined with phloroglucinol-HCl in previous studies was confirmed here with additional positive tests with chlorine-sulfite and toluidine blue. The observation that crystal violet-erythrosin B and safranin-fast green did not detect the presence of lignin until at 72 hr and longer suggests that phloroglucinol-HCl, chlorine-sulfite, and toluidine blue may be staining ligninlike materials rather than lignin. These ligninlike materials were apparently not identical to the lignin present in xylem of the exocarp, which was positively stained with both crystal violet-erythrosin B and safranin-fast green. Positive staining with both phloroglucinol-HCl and chlorine-sulfite did identify the presence of cinnamaldehyde and syringyl groups, respectively (22). Suberin and cutin have not been detected in healed Valencia exocarp (3,4).

Tissue healed for 30 hr was less susceptible to cell wall degradation by exo-PG from *P. digitatum* than was fresh tissue. Also, the cohesiveness of tissue healed for 48 hr was greater than that of fresh tissue following treatment with exo-PG. From these observations, it is apparent that healed tissue is more resistant to enzyme dissolution than is unhealed tissue. Healed tissue became more resistant to mechanical pressure with time. However, the strength of the tissue was not as great as that of unhealed tissue, which hardened after dehydration, but remained susceptible to *P. digitatum*. Increased resistance of cell walls to mechanical penetration after lignification apparently is less important in the resistance of healed tissue than some of the other factors. Garrod et al (9) suggested that antifungal substances associated with healing were more important in resistance than an increase in structural barriers.

Changes observed within the fine structure of hyphae of *P. digitatum* inhibited at healed surfaces suggested that one or more components of the host cell diffused into the fungal cells and disrupted their functions. Organelles were destroyed and replaced with dense protoplasm, and hyphal cell walls were distorted. It seemed unlikely that these changes were induced by the lack of sufficient moisture or nutrients to maintain fungus growth. More moisture was actually present in healed exocarp resistant to penetration by *P. digitatum* than in susceptible unhealed tissues, and the addition of nutrients with spores did not enhance the development of *P. digitatum* in tissue of healed injuries.

The accumulation of phenolic and ligninlike materials in healed Valencia exocarp may be the major factor in resistance to penetration by *P. digitatum*. These materials may be toxic to the invading hyphae, or they may render healed tissue less vulnerable to degradation by macerating enzymes. Poor growth by the pathogen because of the presence of toxic materials would most certainly limit enzyme production. In previous studies (11), phenolic extracts from healed Valencia oranges did not inhibit germination of *P. digitatum*. However, phenolic and ligninlike substances are confined only to cytoplasm and walls at the injured surfaces (4). Concentrations of these substances could easily be diluted during tissue removal and extraction, and amounts tested in vitro may not have been comparable to that in situ. Finally, we have not excluded the possibility that the hyphae of *P. digitatum* become impregnated with ligninlike materials during penetration and lose the plasticity necessary for growth. The potential for hyphal lignification in host resistance has recently been suggested (10).

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