Controlled Heating of Root-Pruned Dormant *Prunus* spp. Seedlings Before Transplanting to Prevent Crown Gall

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ABSTRACT

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Incidence of crown gall was reduced by heating pruned root systems of dormant seedlings of three *Prunus* spp. in Styrofoam ice chests at 18–25 C for 1–3 wk before inoculating the seedlings with *Agrobacterium tumefaciens*. Gall incidence on mahaleb cherry roots was reduced from 100% (unheated control) to 0% after 7 days at about 25 C; the reductions for mazzard cherry and myrobalan plum seedlings were only to 66 and 61% from 100 and 73%, respectively. Later experimentation was conducted only with myrobalan plum seedlings in insulated boxes that provided more uniform and consistent temperature control. In the improved heat boxes, gall incidence dropped from 60% for the unheated myrobalan seedlings to 5% when the seedlings were heated at 24 C for 7 days. At 18 C, 3 wk of heat exposure was required to reduce the incidence of gall to 11%. Gall incidence was reduced further when seedlings were inoculated with the biocontrol agent *A. radiobacter* K84 before exposure to heat. Incidence of gall was not influenced by treatment with indolebutyric acid (IBA, 500 μg/g in methanol and water) as a possible stimulator of wound callus, but seedling mortality reached 90% when IBA treatments were combined with heat exposure. In the absence of IBA, mortality of myrobalan seedlings appeared random across treatments from 2 to 24 C. Holding heat boxes in a cold room at 2–4 C during temperature treatments kept buds from breaking dormancy. The heating procedure was modified for use in a commercial nursery, and the incidence of naturally occurring crown gall on mazzard cherry seedlings was reduced from 66 (unheated) to 6%.

Additional key words: biological control

Woody nursery plants produced in the Pacific Northwest typically are grown from seed for one season, harvested, stored over winter, root-pruned, and replanted in the spring. Seedlings produced in this manner frequently develop crown gall, whereas seedlings grown in the same way but undercut with a digger blade and left in place rather than being harvested develop much less crown gall. The pruning wounds appear to provide infection courts because the galls usually develop at one of these cuts, especially on the taproot. The wounds need protection until sufficient healing can occur to render the wound unsusceptible to infection by *Agrobacterium tumefaciens*.

Numerous preplanting chemical treatments have been attempted to protect these wounds (literature reviewed in 6), largely without success. One of the most successful modern methods of protection has been the biological control of crown gall by *A. radiobacter* strain 84 (4,8). However, this strain is usually ineffective against strains of *A. tumefaciens* that are resistant to the bacteriocin, agrocin 84, and we have continued to look for additional ways to protect susceptible plants.

Our earlier experiments indicated that exposure of the pruned root area to heat might reduce wound susceptibility and thus provide another approach to protection at the time of transplanting. For example, wounds on dormant mazzard cherry seedlings remained susceptible to infection by *A. tumefaciens* for at least 107 days when stored over winter in an unrefrigerated warehouse, but the duration of susceptibility diminished when storage temperatures increased (7). Similarly, the susceptibility of wounds made at the crowns of seedlings grown in the nursery during the hot summer months dropped off markedly by the sixth day after wounding; reduced susceptibility was accompanied by the appearance of callus tissue at the wound. Thus it appeared that the susceptibility of pruning wounds could be reduced by applying heat to the root-pruned dormant seedling, provided the vegetative buds were not stimulated to break dormancy. Howard and Hildreth (3) reported that only a few terminal scion buds had begun to break on apple piece grafts stored for 30 days at 21 C.

Previous research has shown that elevated temperatures enhance wound callusing and rooting of several plant species. For example, the optimum temperature for callus formation in a number of apple cultivars varied from 20 to 25 C (9), whereas Swingle (11) reported 28 and 29 C as for optimal for callus formation on apple and willow cuttings, respectively. Howard and Hildreth (3) observed that callus formed around the graft unions of several apple cultivars within 10 days of wounding if plants were held at 21 C, but 20 days were needed at 13 C. Cellular changes in the wound can be observed much earlier; the first oriented cell division near wounds in *Kalanchoë* occurred 36 hr after wounding in plants maintained at 25 C (5). This time was shortened by 12 hr if plants were maintained at 32 C and by 18 hr at 36 C.

The objectives of this work were to devise a method of applying controlled heat uniformly to freshly pruned root systems of dormant *Prunus* spp. seedlings and to determine whether such a treatment could reduce the susceptibility of dormant seedlings to infection by *A. tumefaciens*.

MATERIALS AND METHODS

Plant materials and cultural procedures. Three species of dormant *Prunus* seedlings (no. 1 grade, 4.8–6.5 mm stem diameter) produced at a commercial nursery were used: *Prunus avium* (Linn.) (mazzard cherry), *P. mahaleb* (Linn.) (mahaleb cherry), and *P. cerasifera* (Ehrh.) (myrobalan plum). Taproots were pruned off 13–15 cm from the crown, and lateral roots were pruned off 0.6–0.9 cm from the main root as practiced commercially. The seedlings were then stored for 24 hr before treatment in clean, moist wood shavings at 2–4 C.

After the seedlings were treated (as described later), groups of five or eight were planted in 3.78-L black plastic pots containing a planting medium of unsterilized sand, peat moss, and natural loam soil (1:1:3, v/v). The seedlings were grown in a greenhouse with supplemental fluorescent lighting for a photoperiod of 14 hr with temperatures of 24 ± 2 C during the day and 20 ± 1 C at night. Each pot of seedlings was topdressed every 3 wk with 150 ml of quarter-strength 20-20-20 (NPK) commercial fertilizer (0.6 g/L of water), grown for 3 mo, harvested, and examined for crown gall. Seedling

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mortality and number of galled seedlings were recorded.

Bacterial strains. A mixed population of four strains of biovar 2 of *A. tumefaciens* (Smith & Towns.) Conn. (strains K27, K29, Q51, and B234) was used routinely as inoculum. These strains were sensitive to agrocin 84 and were obtained as follows: K27 and K29 from A. Kerr, University of Adelaide, Australia; B234 from J. DeVay, University of California, Davis; and we isolated Q51 from a galled cherry tree in Oregon. Individual strains were grown in a yeast-dextrose-peptone broth (7) and centrifuged, then the pellet was resuspended in sterile distilled water (pH 7) and adjusted to a Klett density of 40. Equal amounts of each strain were mixed together for a final concentration of 8.6×10^6 to 4.6×10^7 colony-forming units (cfu) per milliliter. When *A. radiobacter* strain 84 was used, it was grown and processed in the same manner as the pathogens, but the population was adjusted to exceed that of the pathogens by five to 10 times.

Seedlings were inoculated by dipping the treated root and crown into an aqueous suspension of bacteria just before potting the seedlings, except when strain 84 was used in conjunction with indolebutyric acid (IBA). When the seedlings were treated with IBA (500 μg in equal parts methanol and water), they were dipped first in IBA, then the root and crown were sprayed to runoff with K84.

In some treatments, assays were made of K84 populations on plant roots before and after the heat treatment to determine whether the viability of K84 was affected. The crown and root systems were cut from three seedlings and assayed individually by comminuting the tissues in a Waring Blendor in 100 ml of water for 5 min, diluting 1 ml of the suspension serially, and spreading three 0.1-ml aliquots on a medium selective for *A. tumefaciens* biovar 2 (9). Colony counts were expressed as a mean for the three seedlings.

Heat boxes. The first heat boxes were made by arranging thermostatically controlled heating cables over a layer of moist wood shavings in the bottoms of Styrofoam ice chests, covering the cable with additional shavings, standing the seedlings upright over this layer, and adding more shavings around the seedlings to just above the crown. A layer of soil 3 cm thick was placed on top of the shavings to reduce heat loss, and the box and trees were placed in a walk-in cooler at 2–4 C to keep the stems cool and prevent budbreak while the roots were exposed to heat.

Because temperature was not maintained uniformly in the Styrofoam ice chests, the results were irregular and an improved insulated heat box designed by Bhella and Roberts (1) was used the following year. Instead of wood shavings, a mixture of one part fine peat moss and one part plasterer's sand, 10–11 cm deep, was used. Root-pruned seedlings were placed in this medium at an angle of 45–50 degrees to a level just above the crown. The medium was sprinkled lightly with water every 2 days to maintain moisture near field capacity. The boxes with seedlings were kept in a cold room at 2–4 C during the heat treatment to keep the stems cool and retard budbreak.

Treatments and controls. Seedlings with pruned root systems were arranged in heat boxes with temperature settings of 25 C in the first experiments and 18 and 24 C in later experiments. Twenty to 32 root-pruned dormant seedlings were used per treatment. Seedlings were arranged singly in each of two heat boxes, and groups of 40 or more seedlings were removed from each box after 1, 2, or 3 wk at 18 or 24 ± 1.5 C. Each set of 40 or more seedlings was randomly divided into two equal groups; one group was inoculated with *A. tumefaciens* and one group was left uninoculated. All seedlings were then potted and grown as described earlier for 3–8 mo before assessment.

To test whether IBA could increase callusing and reduce wound susceptibility additively with heat, IBA at 500 μg/g in methanol and water (1:1, v/v) was sprayed to runoff over the pruned area of the dormant seedlings, and they were exposed to heat as described.

A further concern during heat treatment was the potential infection of the newly wounded seedlings by epiphytic *A. tumefaciens* that can overwinter on roots. Therefore, root-pruned seedlings were sprayed to runoff with a concentrated suspension of *A. radiobacter* K84 (1–3.5 $\times 10^8$ cfu/ml) mixed with IBA, 500 μg/g. These seedlings were placed in the heat boxes as described before to determine whether K84 would survive and protect the pruning wound during the temperature treatment, especially in the presence of IBA. In subsequent experiments, seedlings were dipped only in K84 (3×10^8 cfu/ml), then exposed to heat.

Control treatments consisted of the same numbers of seedlings that were 1) root-pruned, immediately inoculated with *A. tumefaciens*, and potted; 2) root-pruned and immediately potted without inoculation; and 3) root-pruned, held at 2–4 C for 3 wk, inoculated with *A. tumefaciens*, and potted. All seedlings were placed in the greenhouse immediately after planting.

Use of heat treatments in a commercial nursery. A heating cable (T. I. Corp., Johnson City, TN) thermostatically set for 20 ± 0.5 C (White Rogers with remote sensor from Teufel Nursery, Inc., Portland, OR) was arranged over a layer of moist sawdust 2.5–3 cm deep in a wooden palette 1.2 × 1.8 m with sides 0.3 m high constructed from plywood 1.9 cm thick. Moist sawdust was placed over the heating cable, and dormant mazzard seedlings with the main root pruned at a point 15 cm from the crown were bundled in groups of 50 and set upright in the box.

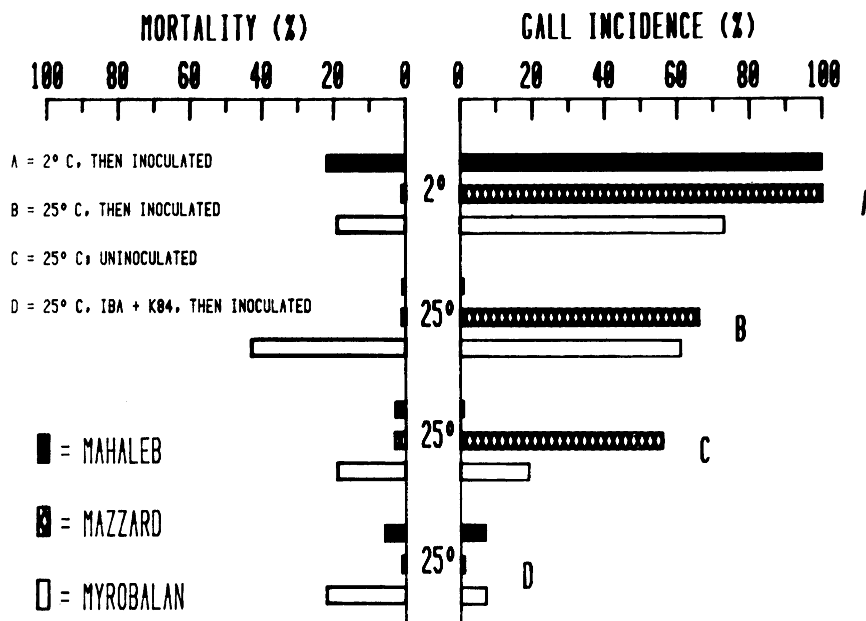

Fig. 1. Effects of elevated temperatures applied to roots of dormant seedlings of three *Prunus* spp. on incidence of crown gall and subsequent mortality of the treated seedlings. A = percentage of living, galled seedlings, of 32 harvested after 3 mo of growth in the greenhouse. B = percentage of 32 seedlings dead at the end of the experiment. C = root-pruned seedlings were held at 2–4 C in a cold room or the root system was heated at 25–34 C for 7 days in a modified Styrofoam ice chest. Roots were then inoculated with 4.5×10^7 cfu of *Agrobacterium tumefaciens* per milliliter (except uninoculated controls) and potted in unsterile planting medium. D = a mixture of indolebutyric acid (500 μg/g) and $1\text{–}3.5 \times 10^8$ cfu of *A. radiobacter* K84 per milliliter was sprayed to runoff over the pruned root system of each seedling before heat treatment. Seedlings were dipped in 4.2×10^7 cfu of *A. tumefaciens* per milliliter just before potting.

Moist sawdust was packed between the bundles to a depth of 10–13 cm above the crown for a total sawdust depth of 25–28 cm. The palette and trees were placed in a cooler at 2–4 C, and the root systems were exposed to heat for 3 wk. Seedlings were then planted in a nursery field in early May, grown according to regular nursery practices for one season, harvested in early October, and assessed for crown gall. Test seedlings were monitored for mortality during the growing season.

RESULTS AND DISCUSSION

Incidence of crown gall was reduced when root-pruned dormant seedlings of all three *Prunus* spp. were exposed to elevated temperatures for at least 1 wk (Figs. 1 and 2). The type of heat chamber was critical to maintain uniform temperatures, and the data from early experiments showed considerable variability because of nonuniform temperatures.

Heat treatments in Styrofoam ice chests. Incidence of crown gall on mazzard and mahaleb cherry and myrobalan plum was reduced after heat treatment. The most striking reduction was 100% for mahaleb (Fig. 1). In contrast, gall incidence was reduced from 100 to 66% and from 73 to 66% for mazzard and myrobalan seedlings, respectively, when inoculated with *A. tumefaciens* after heat treatment compared with counterpart seedlings held at 2–4 C for 7 days before inoculation. Callus was visible on some of the wounds, but it was variable and seemed more abundant on seedlings located near the walls of the chest than on those in the center. Temperatures measured 10 cm below the surface at different locations within the heat boxes ranged from 24 to 34 C, which probably accounted for the variability in gall incidence and wound callusing.

Seedling mortality after heat treatment was minimal for mazzard and mahaleb cherry seedlings but not for the myrobalan plum, which had a high of 43% mortality in one test (Fig. 1). The unexpected temperature of 34 C may have contributed to this high mortality; however, there was some mortality of myrobalan seedlings in each test (Fig. 1), which suggests a low heat tolerance of this species.

The incidence of galled mazzard seedlings in the uninoculated control (55%, Fig. 1) was higher than expected for uninoculated plants, which usually have less than 10% natural infections. This higher incidence of crown gall may reflect a natural microflora of *A. tumefaciens* on the roots, contamination of the medium in the heat box, or contamination before potting the seedlings. It appeared that the problem was not contamination of the medium by *A. tumefaciens*, because corresponding gall incidence in the other two species was less than 50%. There may have been epiphytic colonization of the roots by *A. tumefaciens*, because infection was prevented by K84 (Fig. 1).

Treatment of all three plant species with K84 plus IBA and heat greatly reduced the incidence of infection (Fig. 1). Seedling mortality was also low. Apparently, K84 or IBA contributed more significantly than heat to the reduced level of disease in the mazzard and myrobalan seedlings, because heat alone was less effective in the other tests for these species.

Heat treatments within improved temperature boxes. Incidence of crown gall dropped from 60% (unheated seedlings) to 5% when myrobalan plum seedlings were heated at 24 C for 1 wk before being inoculated with *A. tumefaciens* (Fig. 2B). This low level of infection was sustained throughout the second and third week of exposure at 24 C. In contrast, seedlings treated at 18 C required 3 wk of exposure before gall incidence dropped to 11%. After gall incidence had dropped to 25% after 1 wk of exposure at 18 C, it increased to 66% at the end of 2 wk of exposure at 18 C. This increase was also observed with seedlings that were treated with *A. radiobacter* K84 and heated at 24 C but to a lesser degree (10%) (Fig. 2A). The reason for this rise in infection midway through the heat treatment is unknown. However, Lipetz (5) showed that 1) the time required for the first mitotic cell division (accompanying the wound-healing response) was linearly dependent on temperature, 2) plants maintained at a higher temperature reached a peak of sensitivity to the "tumor-inducing-principle" more rapidly than at low temperatures, 3) that the wound-healing process and the conditioning process (sensitizing the plant to infection) were correlated, and 4) cells exposed before or after the peak of conditioning formed small, slowly growing tumors. The wounds of plants maintained at 18 C in this study may have reached their peak of sensitivity by the second week of heat exposure, thus accounting for the upsurge in galled seedlings.

The incidence of crown gall was 45% when seedlings were wounded and held at 2 C for 3 wk before inoculation, versus 60% when seedlings were inoculated immediately after wounding (Fig. 2B). Susceptibility of wounded seedlings held at 2 C was apparently reduced because the concentration of *A. tumefaciens* was kept constant for the inoculations at 0 and 3 wk. It is doubtful that any appreciable callus tissue formed at the wound, because apple grafts never showed callus formation after 91 days at 2 C (3). However, the wounds may have

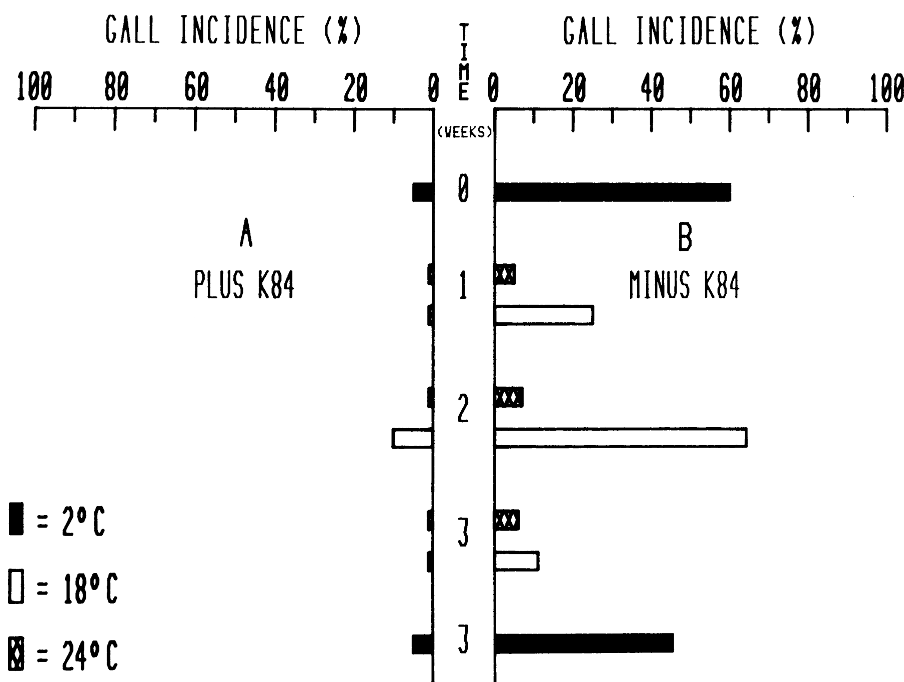

Fig. 2. Incidence of crown gall in relation to exposure of root-pruned myrobalan plum seedlings to elevated temperatures in an improved heat box and inoculation with *Agrobacterium radiobacter* K84 to decrease wound susceptibility to *A. tumefaciens*. Root-pruned seedlings were held at 2–4 C in a cold room for 0 or 3 wk before inoculation, or the root systems were exposed to 18 or 24 C (±2 C at a depth of 3–4 cm) bottom heat in controlled-temperature boxes for 0, 1, 2, or 3 wk while the tops were held at 2–4 C. Groups of 40 seedlings were removed for each treatment and time; 20 were inoculated with *A. tumefaciens* (4×10^7 cfu/ml) and 20 were not inoculated. Each bar represents the percentage of living, galled seedlings among those harvested after 3 mo growth in the greenhouse. **(A)** Seedlings dipped in 1.2×10^8 cfu/ml of strain K84 before exposure to heat or *A. tumefaciens*. **(B)** Seedlings not inoculated with K84. None of the uninoculated seedlings were galled at the end of the experiment. Seedlings were potted in an unsterile soil mix, grown 3 mo in the greenhouse, harvested, and disease incidence assessed.

become colonized by other microbes during the 3-wk storage time, thus altering the suitability of the infection court for *A. tumefaciens* (2).

Infection was completely inhibited when K84 was combined with heat treatment at 24 C compared with a 5% incidence of crown gall when seedlings inoculated with K84 were held at 2–4 C (Fig. 2B). At all temperatures, there was less infection when K84 was used than when it was not. One concern had been that K84 might be restricted by the elevated temperatures, but instead, these data (Fig. 2) suggest that biological control was enhanced. The mean population of K84 per gram fresh weight of root was 3.3×10^6 cfu/g before heating and 7×10^6 cfu/g after heating.

Effect of IBA on susceptibility. There was a significant drop in the incidence of infection after treatment of root-pruned myrobalan plum seedlings with IBA at 500 μg/g, from 70% at zero time to 31% after 3 wk at 2 C (Fig. 3). However, this reduction is very similar to that shown in Figure 2 for seedlings held at 2 C but not treated with IBA, suggesting that IBA had a negligible effect on wound susceptibility. The reduced incidence of crown gall in the earlier test when a mixture of IBA and K84 was applied to seedling roots (Fig. 1) was probably due only to the K84. Evidence supporting this conclusion is presented in Figure 3, where gall incidence was 5% for seedlings inoculated with K84 and *A. tumefaciens* versus 70 and 31% for seedlings treated with IBA and inoculated only with *A. tumefaciens*.

Treatment of myrobalan seedlings with IBA at 500 μg/g was phytotoxic even at 2 C but especially so at 18 and 24 C (Table 1). Because of this, treatment effects on gall incidence were unclear. For example, incidence of gall after 3 wk at 18 and 24 C was 12 and 0%, respectively, but seedling mortality was 60 and 90%, respectively.

Heat treatment and seedling mortality. Mortality was greatest among the myrobalan plum seedlings treated with IBA and heated at 18 and 24 C (Table 1). Overall, phytotoxicity was least for myrobalan seedlings inoculated with K84 and exposed to 18 and 24 C. We had expected that seedling mortality might be greater as the temperature and duration of exposure were increased; however, mortality was random across all temperature treatments. Holding the heat boxes at an ambient temperature of 2 C while heating the pruned roots at 18 and 24 C kept the buds dormant. Thus seedling mortality apparently was not influenced by a premature foliar demand for water (after growth began in the pots) that exceeded the capacity of the developing root system. Maintaining standardized moisture levels in the peat-plasterer's sand mixture was imprecise, and some of the seedlings that died may have been stressed by desiccation during the heat treatment.

Use of heat in a commercial nursery. Incidence of naturally occurring crown gall on 50 heated and 50 unheated mazzard cherry seedlings harvested from field rows was 6 and 66%, respectively. Seedling mortality did not exceed 3%. Several procedures used in the laboratory/greenhouse tests were necessarily altered to comply with commercial nursery routines. Seedlings to be heated were bundled in groups of 50 rather than singly, and sawdust rather than the mixture of peat and sand was arranged around the bundles. However, the sawdust was piled 13–15 cm higher above the seedling crown, which probably helped stabilize the temperature and maintain adequate moisture levels around the bundled seedlings.

The reduced susceptibility of heated *Prunus* spp. seedling roots to infection that has been demonstrated in this paper is attributed to accelerated wound healing and is consistent with other systems where elevated temperatures were used to improve wound healing by callus formation or to prevent infection by *A. tumefaciens* (3,5,9,10). Because the *Prunus* spp. seedlings used in this experiment were produced commercially and are not harvested until December or later, it seemed reasonable to keep the stems cooled during exposure of the root systems to heat. This precaution may have been more stringent than needed. Scion buds on apple grafts remained dormant during the warm period of graft healing (3). However, the apple scion wood had to be collected before onset of

Table 1. Mortality of root-pruned, dormant myrobalan seedlings after treatments of heat, indolebutyric acid (IBA), and *Agrobacterium radiobacter* K84

Temperature (C)[a] and treatment material	Seedling mortality (%)[b] (exposure time [wk])			
	0	1	2	3
2	NT[c]	NT	2	NT
18	10	20	8	NT
24	0	22	12	NT
2 + K84	0	NT	NT	2
18 + K84	NT	0	5	0
24 + K84	NT	0	5	20
2 + IBA	45	NT	NT	20
18 + IBA	NT	70	65	60
24 + IBA	NT	90	75	90

[a] Root-pruned dormant seedlings exposed to 2, 18, or 24 C. In addition, root systems of some seedlings were inoculated with 5.7×10^8 cfu/ml of *A. radiobacter* K84 or sprayed to runoff with IBA at 500 μg/g before temperature treatment.

[b] Each value is the percentage of seedlings dead after 3 mo in the greenhouse of 20 seedlings per test per time period.

[c] NT = not tested.

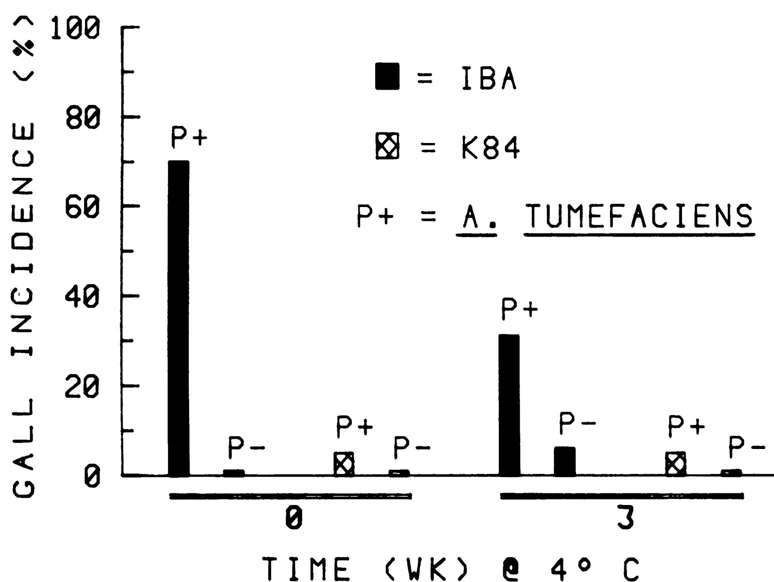

Fig. 3. Effect of indolebutyric acid (IBA) at 500 μg/g and *Agrobacterium tumefaciens* K84 on incidence of crown gall on unheated root-pruned myrobalan plum seedlings inoculated with *A. tumefaciens* or not inoculated after inoculation with *A. radiobacter* K84. P+ and P− represent seedlings inoculated and not inoculated with *A. tumefaciens* (4×10^7 cfu/ml), respectively, after 0 or 3 wk at 2–4 C. Groups of 40 seedlings were removed for each treatment and time; 20 were inoculated with *A. tumefaciens* and 20 were not inoculated. Each bar represents the percentage of living, galled seedlings among those harvested after 3 mo of growth in the greenhouse. Inoculum concentration for K84 was 4×10^8 cfu/ml.

any cold weather that would meet requirements for breaking bud dormancy. Conversely, if heat treatments were applied to entire plants before their buds had sufficient chilling to break dormancy, they would need additional storage time at low temperatures.

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