Drought Tolerance of Tissue-cultured Seedlings of Mountain Laurel (*Kalmia latifolia* L.) Induced by an Endophytic Actinomycete

I. Enhancement of Osmotic Pressure in Leaf Cells

Sachiko Hasegawa, Akane Meguro, Tomio Nishimura* and Hitoshi Kunoh

Institute of Biological Process Research, Akatsuka Orchid Co. Ltd.
1868-3 Takanoo-cho, Tsu, Mie 514-2293, Japan

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Tissue-cultured seedlings of mountain laurel (*Kalmia latifolia* L.) were treated with strain AOK-30 of *Streptomyces padanus* in flasks. This treatment accelerated pigmentation of the seedlings. The untreated green seedlings wilted in room conditions within 1 hr of removal from the flasks while the treated reddish seedlings were still turgid. Fresh weight of leaf pieces decreased more quickly in the untreated than in the treated specimens, suggesting that the AOK-30 treatment somehow rendered the seedlings drought-tolerant. Protoplasts were released from untreated green seedlings with cellulase and macerozyme, while xylanase was required in addition to cellulase for protoplast preparation from the treated reddish seedlings, suggesting that composition and structure of the cell walls of seedlings may have been modified by the AOK-30 treatment. Osmotic pressure was higher in protoplasts from the treated than the untreated seedlings. Elevation of osmotic pressure in cells and cell wall modification may partially account for the enhanced drought-tolerance of the AOK-30-treated seedlings.

INTRODUCTION

The majority of actinomycetes survive in soil as saprophytes by degrading organic materials as nutritional sources. However, since Matsukuma *et al.*¹⁾ and Okazaki *et al.*²⁾ reported that a variety of actinomycetes inhabit a wide range of plants as either symbionts or parasites, endophytic actinomycetes have attracted researchers' attention as potential sources of novel antibiotics and physiological activators^{3,4)}. A number of actinomycetes isolated from soil and/or rhizosphere have been used for agricultural purposes as promising biocontrol agents^{5,6,7)}. Shimizu *et al.*^{8,9,10)} developed unique methods to use endophytic actinomycetes as biocontrol agents: they isolated *Streptomyces galbus* strain R-5, that has broad antimicrobial activity, and added it to flasks in which tissue-cultured seedlings were growing to successfully produce disease-resistant seedlings of rhododendron. Igarashi *et al.*^{3,11,12)} discovered novel antibiotics and growth regulators for plants and animals from several species of actinomycetes of plant origin. These earlier data show that careful investigation of the physiological potential of endophytic actinomycetes could aid in the development of novel technology to enhance human life.

Similar to Shimizu *et al.*⁹⁾, Nishimura *et al.*¹³⁾ isolated a strain of *Streptomyces* sp. AOK-30 [its species was identified as *padanus* recently (unpublished)], from potted mountain laurel (*Kalmia latifolia* L.) based on its antifungal activity. In this paper, we report drought-tolerance of

tissue-cultured seedlings of *Kalmia* that is induced by the use of AOK-30.

MATERIALS AND METHODS

Plant and Actinomycetes

Tissue-cultured seedlings of mountain laurel (*Kalmia latifolia* L., cultivar Ostbo Red) that were growing in flasks were used throughout. Strain AOK-30 of *Streptomyces padanus* had been isolated from potted plants of *K. latifolia*¹³⁾. A spore suspension (500 ml) of AOK-30 in 10% glycerol supplemented with dimethyl sulfoxide (DMSO) was transferred from the stock culture to 100 ml of IMA-2 liquid medium and incubated on a rotary shaker at 100 rpm at 30°C for 24 hr. One ml of a mycelial suspension ($3 \times 10^6 - 4 \times 10^6$ cfu/ml) prepared from this culture was spread over the surface of the rooting medium in glass flasks supporting mountain laurel seedlings that were started from tissue-culture. For untreated controls, 1 ml of IMA-2 liquid medium was spread on the medium surface. All seedlings were then incubated in the incubation room with 12 hr light at 25°C for an additional month.

Frequency of reddish-pigmented seedlings caused by AOK-30 treatment

AOK-30-treated seedlings turned reddish more rapidly than untreated seedlings. To evaluate the degree of pigmentation, the following indices were used: 0 = green stem and leaves; 1 = slightly reddish stem and green leaves; 2

*Corresponding author. Phone: +81-59-230-1234. Fax: +81-59-230-7501. E-mail: tnishimura@akatsuka.gr.jp

= medium reddish stem and 1–5 upper leaves slightly reddish; 3 = intensely reddish stem and 1–6 upper leaves intensely reddish (Fig. 2). Every 10 days after the treatment, 45–60 seedlings were observed with the naked eye, and the degree of pigmentation was scored. Experiments were triplicated, and results were expressed as average indices (Fig. 1).

Examination of loss in fresh weight of leaf segments

As described later, AOK-30-treated reddish seedlings wilted more slowly than untreated green ones when the seedlings were taken from flasks and kept in room conditions for 1 hr (Fig. 3). To confirm this phenomenon, square segments (3.5 mm × 3.5 mm) were cut from the central part of AOK-30-treated reddish and untreated green leaves (10 pieces each) and weighed immediately after cutting. These segments were exposed to room air (20–25°C and 40–45% relative humidity), and their fresh weights were determined every 30 min for 3 hr. Results were expressed as percentages of the initial weight of each segment (0 time) (Fig. 5).

Preparation of protoplasts from leaves

Protoplasts were prepared from AOK-30-treated reddish and -untreated green leaves 10 days after initiation of AOK-30 treatment in flasks as described by Fock *et al.*¹⁴⁾. The epidermis of abaxial surfaces was peeled from 10 leaves with forceps. Leaves were immersed in the enzyme solution(s) in flasks. The cell-wall-degrading enzymes, 2% cellulase (Yakult, Onozuka R-10), 1% macerozyme (Yakult, R-10) and 7.2 mg/ml xylanase (Sigma X-4001) were dissolved singly or in combination in 10 ml of a mixture of 1 M D(–)-mannitol (Wako Chemicals) and 15 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$. After incubation at 27°C for 15 hr, the solution was filtered through double sheets of cheesecloth to

Fig. 1. The degree of pigmentation of tissue-cultured seedlings when the medium surfaces were treated with spore suspension of AOK-30, expressed as average indices. See text for indices.

Fig. 2. The degree of pigmentation of tissue-cultured seedlings. A: Entirely green, B: Upper stems reddish, C: Upper stems reddish and upper leaves partially reddish, D: Upper 5–6 leaves and stems reddish.

Fig. 3. Wilted green (A: untreated with AOK-30) and unwilted reddish tissue-cultured seedlings (B: treated with AOK-30) by 1 hr after removal from flasks.

Fig. 4. Protoplasts released from AOK-30-untreated green (A) and -treated reddish (B) leaves.

remove leaf debris, and then the filtrates were centrifuged at 210 $\times g$ for 5 min to collect the protoplasts. Yield of protoplasts was higher from AOK-30-untreated than -treated leaves.

Examination of osmotic pressure of protoplasts

Protoplasts were suspended in a mixture of mannitol and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ as described. A series of varied concentrations of NaCl were prepared and transferred to Eppendorf tubes (200 μl). A drop of the protoplast suspension (10

Fig. 5. Time course of fresh weight loss from square segments of AOK-30-treated reddish and -untreated green leaves.

ml) was added to the Eppendorf tubes and average number of protoplasts per field of view ($\times 100$ magnification of a light microscope) was determined as that in the original suspension. Within 5 min, the average number of remaining protoplasts per field of view in each treatment was similarly determined. Both counts were repeated in 10 fields of view. Since yield of protoplasts was varied from both leaves as mentioned above, data were analyzed as percentages of average no. of remaining protoplasts versus average no. of protoplasts in the original suspension. Although mannitol was used preliminarily to examine osmotic pressure of protoplasts, mannitol more than 1.4 M was not dissolved in water. Therefore, NaCl solutions were used for this purpose.

RESULTS AND DISCUSSION

Stems and leaves of tissue-cultured seedlings spontaneously turned reddish even in AOK-30-untreated flasks (Fig. 1). Results in Fig. 1 demonstrate that pigmentation of seedlings was accelerated by the AOK-30 treatment. Figure 2 demonstrates the pigmentation pattern from stems to upper leaves: stems and petioles turned reddish more rapidly than the upper leaves. When the medium surface was treated with AOK-30 while the seedlings were growing, approximately 50% of the seedlings turned reddish within 10 days and 70% did so within 30 days. By contrast, only 30% of untreated seedlings turned reddish within 30 days.

Plants express a variety of physiological and morphological responses to external stimuli. Such responses are often associated with activation of the phenylpropanoid pathway, which is involved in flavonoid production associated with pigmentation, phytoalexin, lignification, and so on. Pigmentation of stems of the treated seedlings was seen by the unaided eye within 10 days after the AOK-30 treatment in flasks. A similar acceleration of anthocyanin production was observed in tissue-cultured rhododendron by

Shimizu *et al.*⁸⁾. They suggested that acceleration of anthocyanin deposition might be involved in a systemic acquired resistance to pathogen attack as reported in corn and maize^{15,16)}.

AOK-30-treated and -untreated seedlings were removed from flasks and kept in room air. Untreated green seedlings completely wilted within 1 hr, while AOK-30-treated reddish seedlings remained turgid as shown in Fig. 3A,B. This observation led us to assume that the AOK-30 treatment somehow accelerated drought-tolerance of tissue-cultured seedlings. As indicated in Fig. 5, segments of leaves prepared from untreated green leaves lost fresh weight faster than those from AOK-30-treated reddish leaves; the former lost 70% of its fresh weight, while the latter had lost 50% within 1.5 hr.

These experiments raised another question, whether the osmotic pressure of cells might be enhanced by accumulation of pigments. To address this question, protoplasts were prepared from AOK-30-treated and -untreated seedlings. Cellulase alone released protoplasts only from untreated green leaves but not from AOK-30-treated reddish leaves. A combination of cellulase and macerozyme was more efficient for protoplast release from untreated green leaves, while such a combination also failed to release protoplasts from AOK-30-treated reddish leaves (Table 1). Xylanase, in addition to cellulase, was necessary for successful release from the treated leaves. These results suggest that the AOK-30 treatment modified the composition of the cell walls of the leaves.

When the protoplast suspension was mixed with varied concentrations of NaCl, green protoplasts burst at less than 0.4 M NaCl and shrank at more than 1.2 M. More than 63% of green protoplasts remained in 0.6–1.0 M NaCl solution (Fig. 6). By contrast, more than ca 90% of protoplasts from reddish leaves remained intact in 1.6–1.8 M NaCl (Fig. 6), and a remarkable number of protoplasts still remained in a wide range of NaCl concentrations: 50–60% in 1.2 and 2.0 M and little less than 50% in 1.4 M. The degree of pigmentation was not homogeneous in these protoplasts; some were faintly reddish, but others were deeply reddish. We tentatively assume that such heterogeneity is perhaps associated with a wider range of osmotic pressures in pigmented protoplasts. This suggests that pigment accumulation in vacuoles may be closely associated with elevated osmotic pressure.

Based on these results, protoplasts from AOK-30-treated reddish leaves were concluded to have a higher osmotic pressure than those from untreated green leaves. Therefore, elevation of osmotic pressure in cells may at least partially account for enhanced drought-tolerance. However, we cannot rule out the possibility that structural and compositional modifications of the host cell walls, if any, might be involved in this phenomenon.

Granular chloroplasts were scattered throughout the protoplasts (Fig. 4A), although vacuoles sometimes occupied a large portion of the protoplasts. However, a large red-

Table 1. Release of protoplasts from AOK-30-treated reddish and -untreated green leaves of mountain laurel with cell-wall-degrading enzymes

Enzymes			Approximate number of protoplasts [※] released from	
Cellulase	Macerozyme	Xylanase	Untreated green leaves	Treated reddish leaves
○	○	—	+++	+
○	—	○	++	++
○	—	—	++	—
—	○	○	—	—
—	○	—	—	—
—	—	○	—	—

※+++ : >30 per × 100 view field

++ : 10–20

+ : <10

— : none

Fig. 6. The percentages of protoplasts remaining in various concentrations of NaCl.

- Protoplasts from AOK-30-treated reddish leaves
- Protoplasts from untreated green leaves

dish vacuole occupied the majority of protoplasts from reddish leaves, pushing the chloroplast granules to one end (Fig. 4B), preferentially accumulating anthocyanin in the vacuoles.

Recently, bacterial volatiles have been reported to promote growth¹⁷⁾ and to induce systemic resistance¹⁸⁾ in *Arabidopsis*. Thus, we cannot rule out the possibility that AOK-30 volatiles are associated with this induced drought-tolerance. Minamiyama *et al.*¹⁹⁾ demonstrated that when *Streptomyces galbus* was spread on the surface of the tissue-culture medium where seedlings of rhododendron (family Ericaceae) were growing, mycelia of *S. galbus* grew on leaf surfaces and entered leaf tissues via stomata and that the internal mycelia grew out of stomata after colonizing internal host tissues. In addition, Suzuki *et al.*²⁰⁾ demonstrated that when inoculated *S. galbus* colonized the substomatal spaces of tissue-cultured rhododendron, wall appositions formed in those epidermal and mesophyll cells that had mycelia attached to the outer surfaces. Such wall

appositions indicate that the host cells recognized the presence of this actinomycete and responded to this stimulus. *Streptomyces padanus* that had been applied to tissue-cultured seedlings of *Kalmia*, a member of family Ericaceae, was reisolated from apical leaves of the seedlings within 7 weeks, suggesting successful colonization of this strain in the host tissues²¹⁾. Based on these earlier reports, tissue-cultured seedlings may recognize and respond to these endophytic actinomycetes as an external stimulus. However, it is still too early to say whether specific secondary metabolites and/or volatiles of AOK-30 are involved in induction of the drought tolerance.

Because tissue-cultured seedlings are generally acclimatized under highly humid conditions that are advantageous for survival of pathogens, a variety of diseases often occur during acclimatization. Thus, if drought tolerance of tissue-cultured seedlings is enhanced by actinomycetes, perhaps the seedlings can be acclimatized under a relatively lower humidity, enabling the seedlings to escape disease.

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