Postharvest Berry Drop of Seedless Berries Produced by GA Treatment in Grape Cultivar 'Kyoho' III. Effect of GA on Xylem Differentiation and Lignification of Rachis

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III. Effect of GA on Xylem Differentiation and Lignification of Rachis

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Summary

The production of secondary xylem cells, lignification and phenylalanine ammonia-lyase (PAL) activity in rachis were studied, in comparison of five cultivars, 'Kyoho', 'Campbell Early', 'Muscat Bailey A', 'Delaware' and 'Himrod', to get a key to prevent the rachis hardening induced by gibberellin (GA) treatment especially in 'Kyoho'.

In 'Kyoho' and 'Campbell Early', rachises of which are easy to harden following GA treatment, the number of cells along the radial rows of secondary xylem increased beginning earlier in GA-treated than control rachises, and the difference became greater with time. On the other hand, in 'Muscat Bailey A', 'Delaware' and 'Himrod', rachises of which are difficult to harden following GA treatment, the number of cells was small and differed little between GA-treated and control rachises.

The number of cells along the radial rows of secondary xylem in rachis treated with GA in 'Kyoho' was one-third that in 'Campbell Early'. In 'Kyoho', increase in lignin content in rachis following GA treatment, however, was 2 times that in 'Campbell Early', although in 'Muscat Bailey A' little increase was found. Hence, the hardening of GA-treated rachis in 'Kyoho' seemed to be more largely due to the lignin accumulation in rachis than that in 'Campbell Early', which seemed to be largely due to the increased number of secondary xylem cells.

More phenols dissolved in a liquid medium from segments of cane sampled in February in 'Kyoho' and 'Campbell Early' than in 'Muscat Bailey A' and 'Himrod'. Addition of GA into the liquid medium, however, only slightly increased the production of phenols from cane regardless of cultivars. In a liquid culture of rachis sampled late in May, GA increased phenol production in 'Kyoho' and 'Campbell Early', but not in 'Muscat Bailey A', 'Himrod' and 'Delaware'.

PAL activity in GA-treated rachises in 'Kyoho' and 'Campbell Early' increased beginning a little earlier than lignin accumulation, and was kept higher for a long time than that of the control rachis. It was especially true for 'Kyoho'.

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While, in 'Muscat Bailey A' and 'Delaware', PAL activity was considerably high both in GA-treated and control rachises and it was only for a short time that the PAL activity was kept higher in GA-treated than control rachises.

It was suggested that the control of PAL activity might be an effective method to prevent the rachis hardening induced by GA especially in 'Kyoho'.

We previously showed (1) that the hardening of rachis treated with GA was related to the thickening of rachis, which, in turn, seemed to be caused by the increase of the number of secondary xylem cells, although the extent of lignification of the cells seemed to be also involved. Bradley and Crane (2) reported that spraying spur-shoots of apricot with GA stimulated cambial activity and led to increased xylem development. Shanan (3) reported that treatment of dwarf bean plants with GA produced a characteristic stem elongation accompanied by an increase in lignification of meta and secondary xylem. Using cultured *Coleus* internode slices, Rubery and Fosket (4) suggested that PAL was a "marker enzyme" for xylogenesis and that PAL activity might be a rate limiting step in lignification.

The purpose of this experiment was to elucidate the relationship between GA treatment, the differentiation and lignification of secondary xylem cells in rachis and PAL activity to get a key to prevent the rachis hardening induced by GA treatment.

Materials and Methods

1. *Xylem Differentiation and Lignification Varying with Cultivars.*

Eight-year-old 'Kyoho', 18-year-old 'Campbell Early', 'Muscat Bailey A' and 'Delaware' and 4-year-old 'Himrod' were used. 'Kyoho' and 'Campbell Early' are easy-to-drop cultivars, while 'Muscat Bailey A', 'Delaware' and 'Himrod' are difficult-to-drop cultivars (5). Clusters of each cultivars were dipped into GA_3 solution at 100 ppm with 100 ppm Aerol OP as a wetting agent at full bloom (June 16 in 'Campbell Early' and 'Delaware', June 18 in 'Kyoho' and 'Himrod', and June 19 in 'Muscat Bailey A'). After GA treatment, 10 clusters of each cultivar except 'Himrod', in which 3 clusters were used, were sampled 3 times at intervals of 3 days, and, thereafter, 7 times at intervals of 5 days together with control (GA-untreated) clusters. Rachises were cut off from the clusters, and some of them were stored in 50% alcohol for the determination of the number of secondary xylem cells. The remainder were stored at -20°C for the determinations of PAL activity and lignin content.

1) *Number of secondary xylem cells.* The number of cells along the radial rows of secondary xylem was counted as reported previously (1).

2) *PAL activity.* Acetone powder was prepared by homogenizing 10 g of small pieces of frozen rachis twice for 10 min at 0°C with 50 ml of acetone previously

chilled to -20°C. The homogenates were filtered, rinsed with chilled acetone, and dried for 1hr at room temperature and stored at -20°C until used. PAL activity in the acetone powder was assayed by the method of Rahe *et al.* (6) with some modifications. One g of the aceton powder was extracted with 20 ml of cold 0.1M borate (Na)-HCl buffer, pH 8.8, for 1 hr at 0°C with slow stirring. The suspension was centrifuged at 20,000 × *g* for 20 min at 0°C. The supernatant was used for determination of PAL activity. The reaction mixture contained 2 ml of 0.1M borate (Na)-HCl buffer, pH 8.8, 50 μmoles of L-phenylalanine, and 2 ml of enzyme solution. A blank was employed in which L-phenylalanine was absent until the reaction had terminated. The reaction mixture was incubated for 1 hr at 40°C in a glass tube without agitation. The reaction was terminated by the addition of 0.1 ml of 5N HCl. The acidified mixture was extracted with 7 ml of ether. The aqueous phase was removed, and ether was evaporated to dryness. The residue was dissolved in 2.5 ml of 0.05N NaOH. The concentration of cinnamic acid was determined colorimetrically by absorbance at 269 nm, with 0.05N NaOH as a reference. A unit of activity was defined as the amount of enzyme which catalyzes the production of 1 μmole of cinnamic acid per hr. Soluble protein content was determined by the procedure of Lowry *et al.* (7). PAL activity was expressed as mUnit per mg of protein.

3) *Lignin.* The residue of acetone powder after PAL extraction was used. The residue was dried at 80°C for 2 days and ground. After kept in a desiccator for a day, it was weighed, and 0.5 g of it was extracted with 50 ml of boiling ethanol-benzen (1 : 2 v/v) for 6 hr. Lignin was determined by Klason method (8) and the result was expressed as a percentage increase in lignin content based on dry weight of acetone powder over the time of GA application.

2. *Phenol Production in Liquid Culture.*

1) *Cane.* Eight-year-old 'Kyoho', 18-year-old 'Campbell Early', 'Muscat Bailey A' and 'Delaware', and 4-year-old 'Himrod' were used. The 20-cm canes were cut off in February from each cultivar and stored at 5°C. After sterilized with 0.001% benzalkonium chloride for 2 hr, the canes were dissected into 1.5-cm segments and the epidermis were peeled off. The segments were sterilized with 70% ethanol and then with filtrate of 10% chlorinate lime for 10 min, respective-ly. Then, one segment was put into 20 ml of liquid medium (Murashige and Skoog's medium) in a 50 ml Erlenmeyer flask, sealed with aluminum foil, and incubated on a shaker under the light of 2,400 - 2,700 lux at 25°C. GA$_3$ at the final concentration of 100 ppm was added to each culture medium through millipore filter with 0.45-μm pore size. After 7 days of liquid culture, fresh weight, diameter, and length of the segment were measured and total phenols dissolved into the medium were determined. All experiments were carried out in 5 replications.

2) *Rachis.* The lowest flower clusters on the shoots were sampled from the 5
cultivars mentioned above on May 31.   The 1.5-cm segments of peduncle were
used for liquid culture.

3) *Tendril, rachis and stem.* Four-year-old 'Kyoho' grown in a greenhouse were
used.   Tendrils on the 3rd node from shoot apex, rachis, and stem of the shoot at
flowering were sampled on May 9.   One-cm proximal ends of the lowest branches
of tendrils and rachis, and 1-cm stem segment of the 5th internode from shoot apex
were cultured for 8 days.

4) *Measurement of total phenols.* One ml of culture medium was transferred
from a flask into a test tube with a syringe, and 1 ml of Folin reagent and 1 ml of
10% Na_2CO_3 were added.   The mixture was incubated at 80°C for 2 hr and then
kept at 5°C.   After 12 hr, total phenols were determined .

3. *Measurement of Total Phenol Content of Stem.*

On May 21, stems without bark and about 0.34 g in weight were sampled from
the 5th internodes from the apices of the shoots of the 5 cultivars.   After
measurements of length and diameter, they were separately refluxed with 25 ml of
80% alcohol for 30 min.   The extract was held for 5 hr at room temperature, and
the total phenol content was determined.

Results

1. *Increase in the Number of Secondary Xylem Cells Following GA Treatment
Varying with Cultivars.*

In 'Kyoho', the secondary Xylem cells of rachis treated with GA began to
increase 3 days after the treatment, and the increase continued for about 26 days.
While those of control rachis began to increase 11 days later, and the increase
ceased after 10 days.   Thus, on July 27, the number of secondary xylem cells of
GA-treated rachis was 2 times that of the control rachis.   In 'Campbell Early', the
secondary xylem cells began to increase 3 days after GA treatment.   The increase
temporarily ceased from June 30 to July 15, but a marked increase occurred again
thereafter.   In control rachis, they began to increase simultaneously with GA-
treated rachis, but the increase ceased on June 25.   Thus, in 'Campbell Early', the
number of secondary xylem cells of GA-treated rachis was 4 times that of the
control rachis on July 30.   On the other hand, in 'Muscat Bailey A', the secondary
xylem cells began to increase 3 days after GA treatment but the increase was small
and ceased 6 days later in both GA-treated and control rachises.   There was no
difference in the number of secondary xylem cells between them on July 28.   In
'Delaware', the secondary xylem cells began to increase 6 days after GA treatment
in both GA-treated and control rachises.   The increase continued until 39 and 29
days after the treament in GA-treated and control rachises, respectively, although
it was smaller than in 'Kyoho' and 'Campbell Early'.   There was a little differ-
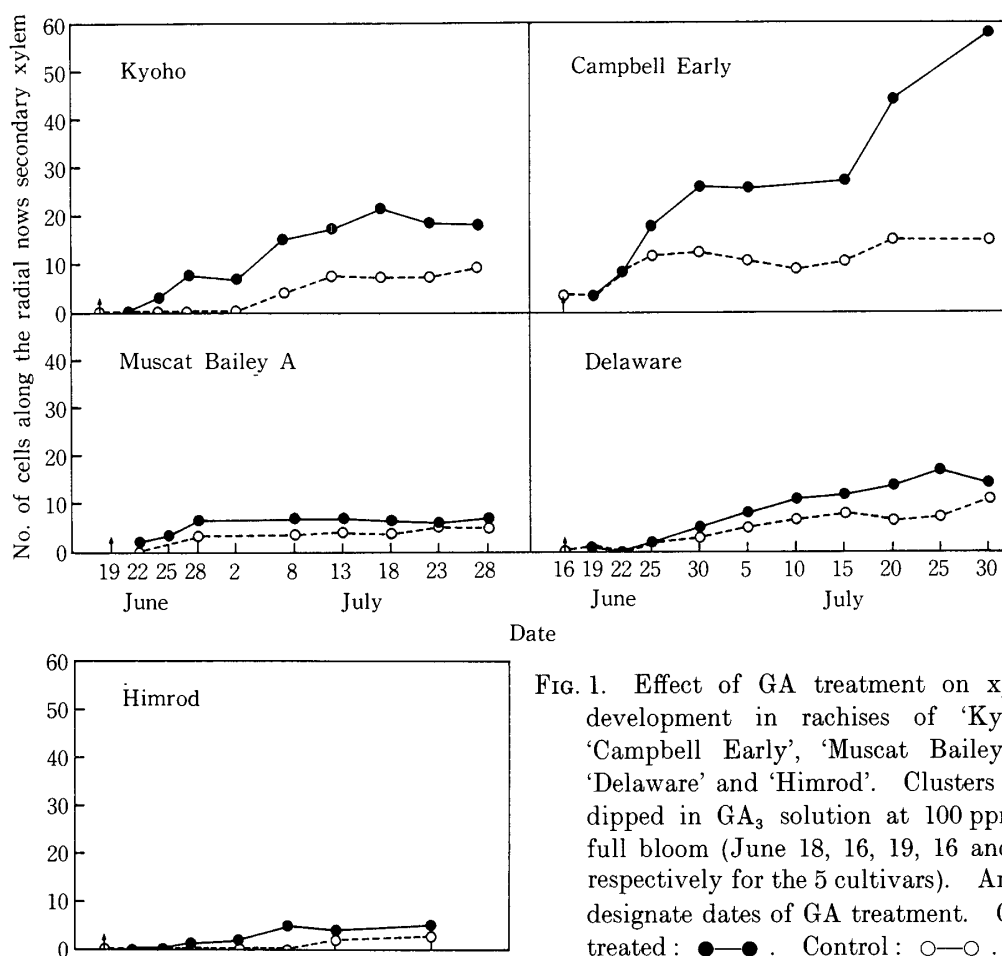
Fig. 1. Effect of GA treatment on xylem development in rachises of 'Kyoho', 'Campbell Early', 'Muscat Bailey A', 'Delaware' and 'Himrod'. Clusters were dipped in GA_3 solution at 100 ppm at full bloom (June 18, 16, 19, 16 and 18, respectively for the 5 cultivars). Arrows designate dates of GA treatment. GA_3-treated : ●—● . Control : ○—○ .

ence in the number of secondary xylem cells between the two rachises on July 30. In 'Himrod', the increase was very small in both GA-treated and control rachises, and differed little between them on July 22 (Fig. 1).

2. *Lignification of Rachis Following GA Treatment Varying with Cultivars.*

In 'Kyoho', there were found two rapid increases in lignin content of GA-treated rachis, beginning 3 and 34 days after GA treatment, respectively. In control rachis, the increase of lignin content began 11 days later than in GA-treated rachis, and had almost ceased by July 22. The increase in lignin content in GA-treated rachis was about 4 times that in the control rachis on August 2. In 'Campbell Early', a rapid increase of lignin content in GA-treated rachis occurred from 9 to 14 days after GA treatment, while, in control rachis, it began 15 days later than in GA-treated rachis, and continued for 10 days. The increase in lignin content in GA-treated rachis was 2 times that in the control rachis on July 30. On the other hand, in 'Muscat Bailey A', the lignin content increased only slighty in both GA-treated and control rachises. In 'Delaware', a rapid increase in lignin content in GA-treated rachis occurred from 14 to 24 days after GA treatment,
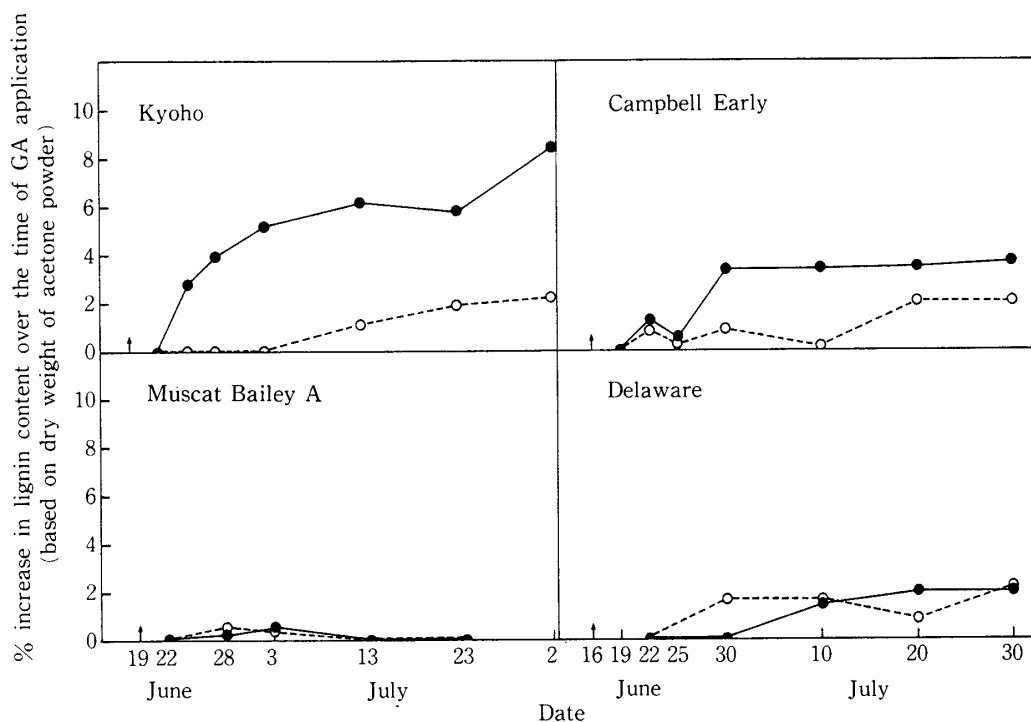
Fig. 2.   Effect of GA treatment on lignin accumulation in rachises of 'Kyoho', 'Campbell Early', 'Muscat Bailey A' and 'Delaware'.   Arrows designate dates of GA treatment. GA₃-treated : ●—● .   Control : ○—○ .

while, in control rachis, from 6 to 14 days after.   However, the increase was small in both rachises and differed little between them on July 30 (Fig. 2).

3.   *Increase in Phenol Production Following GA Treatment Varying with Cultivars.*

Phenol production by cane segments cultured with liquid medium : Phenol production by the cane segments expressed per unit surface area was higher in 'Kyoho' and 'Campbell Early' than 'Muscat Bailey A', Himrod' and 'Delaware'. GA, however, had no effect on the phenol production by the cane segment in any of the cultivars used (Table 1).

Phenol production by rachis segments cultured in liquid medium : With the cane segments, more phenols were produced   per unit surface area by rachis segments of 'Kyoho' and 'Campbell Early' than those of 'Muscat Bailey A', 'Himrod' and 'Delaware'.   Further, GA increased phenol production by rachis segments in 'Koyho' and 'Campbell Early' but not in 'Muscat Bailey A', 'Himrod' and 'Delaware'.   The same trend was shown also on fresh weight basis (Table 2).

Phenol production by tendril, rachis and stem segments cultured in liquid medium : In 'Kyoho', more phenols were produced by tendril and rachis segments than stem segments.   GA stimulated phenol production by tendril, rachis and stem segments (Table 3).

Phenol content of stem segments : More phenols were extracted per unit

TABLE 1. *Effect of GA_3 on Production of Phenols by Cultured Cane Segments in 'Kyoho', 'Campbell Early', 'Muscat Bailey A', 'Himrod' and 'Delaware'.*

		Phenols* (mg)	Fresh wt/ segment (g)	Phenols/ fresh wt (mg/g)	Surface area/ segment (cm²)	Phenols/ surface area (mg/cm²)	Segment width (cm)
Kyoho	GA_3	1.25	0.99	1.26	5.51	0.23	0.9
	None	1.23	0.99	1.24	5.51	0.22	0.9
Campbell	GA_3	1.09	0.90	1.21	5.51	0.20	0.9
Early	None	1.00	0.90	1.11	5.51	0.18	0.9
Muscat	GA_3	0.20	0.27	0.74	2.75	0.04	0.5
Bailey A	None	0.20	0.27	0.74	2.75	0.04	0.5
Himrod	GA_3	0.67	0.78	0.86	5.51	0.12	0.9
	None	0.68	0.78	0.87	5.51	0.12	0.9
Delaware	None	0.61	0.35	1.74	4.78	0.11	0.8

* mg gallic acid equivalent per culture (20 ml).

TABLE 2. *Effect of GA_3 on Production of Phenols by Cultured Rachis Segments in 'Kyoho', 'Campbell Early', 'Muscat Bailey A', 'Himrod' and 'Delaware'.*

		Phenols (mg)	Fresh wt/ segment (g)	Phenols/ fresh wt (mg/g)	Surface area/ segment (cm²)	Phenols/ surface area (mg/cm²)	Segment width (cm)
Kyoho	GA_3	0.21	0.18	1.16	1.81	0.12	0.4
	None	0.14	0.17	0.83	1.69	0.08	0.3
Campbell	GA_3	0.18	0.12	1.52	1.41	0.13	0.3
Early	None	0.13	0.11	1.22	1.51	0.09	0.3
Muscat	GA_3	0.05	0.13	0.39	1.56	0.03	0.3
Bailey A	None	0.04	0.10	0.37	1.42	0.03	0.3
Himrod	GA_3	0.06	0.14	0.43	1.66	0.04	0.3
	None	0.07	0.13	0.54	1.57	0.04	0.3
Delaware	GA_3	0.02	0.04	0.56	1.00	0.02	0.2
	None	0.02	0.04	0.48	0.87	0.02	0.2

TABLE 3. *Effect of GA_3 on Production of Phenols by Cultured Tendril, Rachis and Stem Segments in 'Kyoho'.*

		Phenols (mg)	Fresh wt/ segment (g)	Phenols/ fresh wt (mg/g)	Surface area/ segment (cm²)	Phenols/ surface area (mg/cm²)	Segment width (cm)
Tendril	GA_3	0.08	0.03	2.67	0.62	0.13	0.2
	None	0.04	0.03	1.33	0.62	0.06	0.2
Rachis	GA_3	0.14	0.06	2.33	1.08	0.13	0.3
	None	0.10	0.06	1.67	1.08	0.09	0.3
Stem	GA_3	0.12	0.07	1.71	1.08	0.11	0.3
	None	0.07	0.07	1.00	1.08	0.06	0.3

TABLE 4. *Extraction of Phenols from Excised Stem* in 'Kyoho', 'Campbell Early', 'Muscat Bailey A', 'Himrod' and 'Delaware'.*

	Phenols (mg)	Fresh wt/ segment (g)	Phenols/ fresh wt (mg/g)	Surface area/ segment (cm²)	Phenols/ surface area (mg/cm²)	Segment width (cm)
Kyoho	0.74	0.34	2.18	1.83	0.40	0.4
Campbell Early	0.82	0.34	2.41	1.89	0.43	0.3
Muscat Bailey A	0.66	0.34	1.94	2.02	0.33	0.2
Himrod	0.58	0.34	1.71	1.71	0.34	0.4
Delaware	0.63	0.34	1.85	2.13	0.30	0.2

* The 5th internode from the shoot apex.

weight and unit surface area from stem segments in 'Kyoho' and 'Campbell Early' than in 'Muscat Bailey A', 'Himrod' and 'Delaware' (Table 4).

4. *Increase in PAL Activity Following GA Treatment Varying with Cultivars.*

In 'Kyoho', PAL activity in GA-treated rachis began to increase 3 days after GA treatment, and reached the first and second peaks 9 and 24 days after GA treatment, respectively. The second peak was higher than the first one. While,
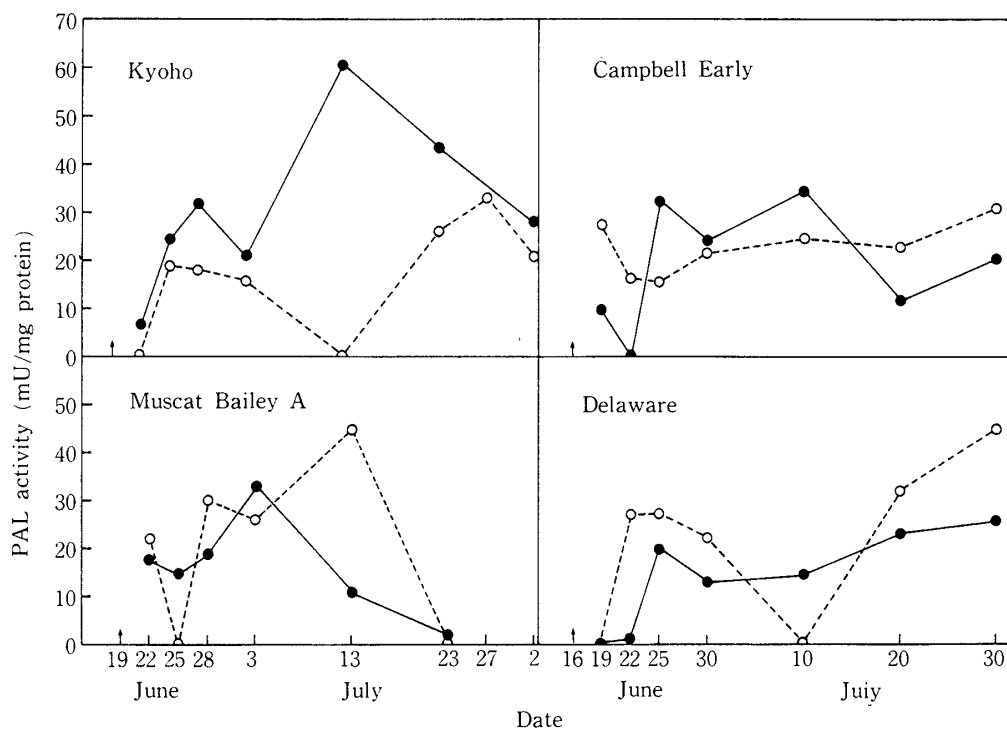

FIG. 3. Effect of GA treatment on phenylalanine ammonia-lyase activity in rachises of 'Kyoho', 'Campbell Early', 'Muscat Bailey A' and 'Delaware'. Arrows designate dates of GA treatment. GA₃-treated : ●—● . Control : ○—○ .

in the control rachis, PAL activity reached the first peak 3 days earlier, then it almost disappeared and reached the second peak 15 days later, although the peaks were lower than in GA-treated rachis. In 'Campbell Early', PAL activity in GA-treated rachis was kept higher than that in the control rachis from 9 to 24 days after GA treatment, while, in control rachis, there was no clear peak and no rapid increase. In 'Muscat Baily A', PAL activity in GA-treated rachis increased from 6 days after GA treatment and reached a peak after 8 days. The activity in the control rachis, however, was also high. In 'Delaware', the rapid increase of PAL activity in GA-treated rachis occurred from 6 to 9 days after GA treatment, and was kept high thereafter. The activity in control rachis, however, was higher than that in GA-treated rachis except 24 days afer the treatment (Fig. 3).

Discussion

In 'Kyoho' and 'Campbell Early', rachises of which are easy to harden following GA treatment, the number of secondary xylem cells increased beginning earlier after GA treatment and the difference from that of the control rachis became greater with berry growth. On the other hand, in 'Muscat Bailey A', 'Delaware' and 'Himrod', rachises of which are difficult to harden following GA treatment, the increase in the number of secondary xylem cells was small and differed little between GA-treated and control rachises. These results showed clearly that rachis hardening following GA treatment was associated with the increase in the number of secondary xylem cells in rachis, as reported previously (1), although the extent of increase differed with cultivars. However, the number of secondary xylem cells in GA-treated rachis was 18 and 57 in 'Kyoho' and 'Campbell Early', respectively. While, the hardness of GA-treated rachis in 'Kyoho' was almost the same as in 'Campbell Early' (5). Accordingly, it appeared that in the case of 'Kyoho', hardening of GA-treated rachis could not necessarily be explained only from the increase in secondary xylem cells.

In 'Campbell Early', increase in lignin content following GA treatment was 4%, 2 times that of the control. While, in 'Kyoho', it was 8%, 4 times that of the control. On the contrary, in 'Muscat Baily A', the increase was small in both GA-treated and control rachises, and in 'Delaware', there was no difference between GA-treated and control rachises, though there was a small increase in both of them. From these results, it seemed that in 'Kyoho', lignification of rachis after GA treatment was more marked than in 'Campbell Early', which resulted in the same degree of rachis hardness as in 'Campbell Early' in spite of a smaller number of secondary xylem cells of rachis.

From segments of canes sampled in February more phenols dissolved in liquid medium in 'Kyoho' and 'Campbell Early' than 'Muscat Bailley A' and 'Himrod'. The production of phenols, however, was affected little by the addition of GA to the liquid medium, irrespective of cultivars. While, when the segments of rachis

were sampled late in May, the phenol production was increased much by the addition of GA in 'Kyoho' and 'Campbell Early', but little in the other three cultivars. Thus, it was apparent that in 'Kyoho' and 'Campbell Early', GA increased phenol production in the segments of tendril, rachis and stem prepared in the stages when the rachis hardened following GA treatment, although it was not true for the samples prepared in the other stages. Further, it was confirmed by determination of phenols in the stem of shoots sampled in May that 'Kyoho' and 'Campbell Early' contained more phenols than 'Muscat Bailey A', 'Himrod' and 'Delaware'.

In 'Kyoho' and 'Campbell Early', PAL activity in GA-treated rachis began to increase a little earlier than lignin accumulation, and it was kept higher for a long time than that in control rachis. It was especially true for 'Kyoho'. While, in 'Muscat Bailey A' and 'Delaware', PAL activity was considerably high both in GA-treated and control rachises and it was only for a short time that the PAL activity was kept higher in GA treated than control rachises. The high activity in the control rachis is not easy to explain, but it might be related to a physiological processes other than xylogenesis and lignification.

Thus, at least in 'Kyoho' and 'Campbell Early', the changes in PAL activity in advance of the lignin accmulation in rachises seemed to show that lignification of rachis induced by GA was controlled by the enzyme, PAL, as indicated by Cheng and Marsh (9) in dwarf pea and Higuchi (10) in bamboo. Consequently, finding out some method to control the PAL activity may become an effective measure to prevent rachis hardening following GA treatment especially in 'Kyoho'

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