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## Phenylalanine Ammonia Lyase Inhibitors-2,5-Norbornadiene and $\alpha$ -Aminooxi- $\beta$ -phenylpropionic Acid Reduce Toughening and Lignification of Stored Asparagus Spears

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**Abstract:** Two inhibitors of the first enzyme phenylalanine ammonia lyase (PAL) in the phenylpropanoid pathway were used to investigate the role of phenolic metabolism in lignification and toughness of Asparagus spears. Spears were soaked for 1 h at 20°C in aqueous solution of  $\alpha$ -aminooxi- $\beta$ -phenylpropionic acid (AOPP) at 0, 100, 300 and 600  $\mu$ M. Liquid 2,5-Norbornadiene (NBD) was applied with a micropipette onto a sheet of filter paper in a 6.4 liter big jar which was immediately sealed with a cover. Liquid NBD readily volatilized at 25°C in a closed chamber. The amount of liquid applied was calculated to give the desired gaseous concentration (6000  $\mu$ l L<sup>-1</sup>) in the jar. Both AOPP and NBD inhibited PAL activity and significantly decreased fiber content in both top and bottom portions of the spears. Northern blot analysis showed that expression in mRNA level coincided well with the changes in PAL activity and fiber content. These results confirm the view that AOPP and NBD, inhibitors of PAL enzyme activity, reduce PAL gene expression and decrease the fiber content of Asparagus spears, suggesting that downstream products of PAL enzyme activity may have a regulatory role in the lignification process.

**Key words:**  $\alpha$ -aminooxi- $\beta$ -phenylpropionic acid, 2,5-Norbornadiene, *Asparagus officinalis*, phenylalanine ammonia lyase, toughness

### INTRODUCTION

The toughening of Asparagus is due to lignification of fibrovascular tissues and occurs within hours after harvest in spears stored at ambient conditions. Lignin, the substance that lends fibers their toughness, is polymerized from cinnamyl alcohols derived from shikimic acid pathway<sup>[1]</sup> and PAL catalyzing the conversion of L-phenylalanine to *trans*-cinnamic acid in this pathway has been considered to play an important role as a key enzyme. Fluctuation in PAL activity has been shown to be a key element controlling the synthesis of lignin and in many cases, an increase in the amount of PAL mRNA has been shown to underlie the increase of PAL activity<sup>[2-5]</sup>. Numerous reports have demonstrated a positive correlation between increased enzyme activity, PAL protein accumulation and PAL gene expression. Inhibition of PAL activity by compounds such as 2,5-Norbornadiene (NBD), 2-aminoindan-2-phosphonic acid (AIP),  $\alpha$ -aminooxyacetic acid (AOA) and  $\alpha$ -aminooxi- $\beta$ -phenylpropionic acid (AOPP) has been well characterized in several plant tissues<sup>[6-10]</sup>.

In our previous experiments we observed an increase in toughness in both top and bottom portions of stored

asparagus spears<sup>[11,12]</sup>. Since toughness in asparagus has been reported to result from increased PAL activity and lignin synthesis<sup>[12,13]</sup> which are induced by wounding, experiments were designed to investigate the effect of inhibitors of PAL on toughness. Our previous report<sup>[12]</sup> showed that induction of PAL activity in harvested Asparagus is regulated by transcription of pAS-PAL in response to the wounding associated with harvest but our Northern blot analysis revealed that the expression of pAS-PAL increased in harvested asparagus until 24 h of storage period at 20°C and afterwards it started to decline although the toughness determined as the value of breaking force was still increasing. Therefore, the objective of this study was to further examine the role of PAL in toughening of stored asparagus spears by using PAL inhibitors like AOPP and NBD.

### MATERIALS AND METHODS

Green asparagus spears (*Asparagus officinalis* L. cv. Welcome) were directly harvested from a commercial crop in Kagawa, Japan. Spears were hand harvested and trimmed to approximately 25 cm length. The spears, which were of good quality, straight with closed bracts, were

soaked for 1 h at 20°C in aqueous solution of  $\alpha$ -aminooxy- $\beta$ -phenylpropionic acid (AOPP) at 0, 100, 300 and 600  $\mu$ M. Liquid NBD was applied with a micropipette onto a sheet of filter paper in a 6.4 L big jar which was immediately sealed with a cover. Liquid NBD readily volatilized at 25°C in a closed chamber. The amount of liquid applied was calculated to give the desired gaseous concentration (6000  $\mu$ l liter<sup>-1</sup>) in the jar. After necessary treatments the spears were put in polyethylene bags and held at 20°C for up to 48 h. Fiber content in both top and bottom portions of the spears was measured at harvest (0 h) and after 12, 24 and 48 h. For PAL enzyme analysis and RNA extraction the spears were frozen at -80°C.

Enzyme extraction and assay, RNA isolation, cloning and sequencing of cDNA and Northern blot analysis for PAL were as previously described<sup>[12]</sup>.

## RESULTS

**Inhibition of PAL activity:** In the control samples, PAL activity started to increase after harvest and continued throughout the experimental period (Fig. 1B and 3B). Both 300 and 600  $\mu$ M AOPP inhibited PAL activity maintaining the level as initial. AOPP at a concentration of 100  $\mu$ M was not effective. At a much higher concentration of 600  $\mu$ M no additional effect but somewhat less inhibition of the PAL activity was observed. Exposure of spears to NBD also resulted in a partial inhibition of the induction of PAL activity. In control spears the PAL activity started to increase after harvest and continued throughout the experimental period, whereas in NBD treated spears suppression of PAL activity started after 12 h of excision and after 24 h NBD suppressed the PAL activity by 64%.

**Expression of PAL gene:** PAL gene expression was detected in both top and bottom portion of the spears beginning just after harvest and continued up to 48 h (Fig. 2 and 4). In case of 100  $\mu$ M AOPP treated spears gene expression followed almost same pattern like the control one. In contrast, for 300 and 600  $\mu$ M AOPP treated spears almost no or very little expression of pAS-PAL was detected that were coincided well with enzyme activity. In NBD treated samples the expression of pAS-PAL also followed the same pattern like enzyme activities. The transcript for PAL was detected after 12 h of excision and showed the maximum expression at 24 h for both control and NBD treated spears. Obviously the level of expression was higher in control than in NBD treated spears.

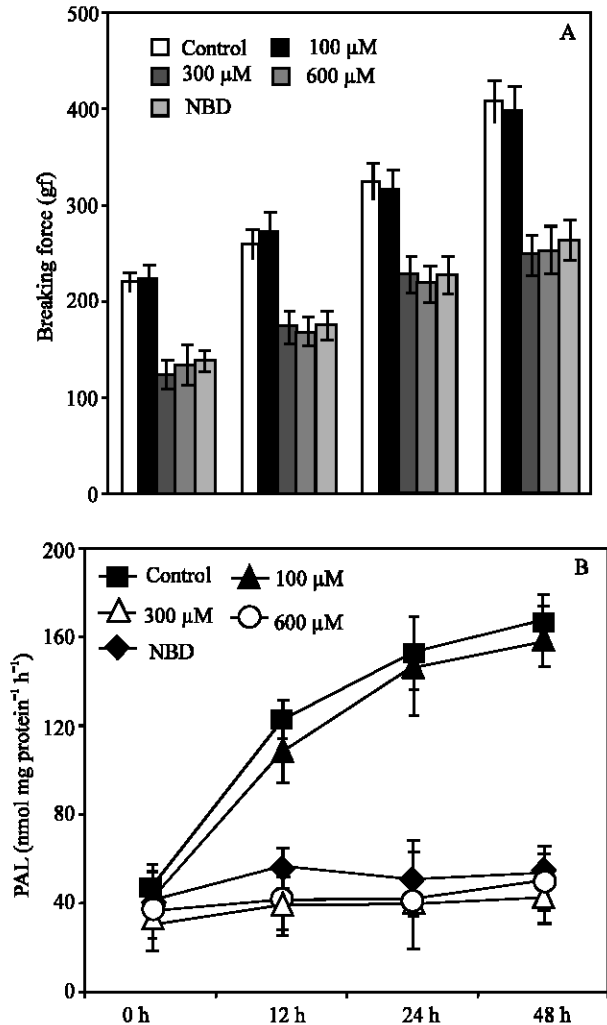


Fig. 1: Effect of AOPP and NBD on fiber content and PAL activity in top portion of asparagus spears. (A) Changes in breaking force. Each point represents the mean of three replicates and vertical bars show SE. (B) Changes in PAL activity. Each point represents the mean of three replicates and vertical bars show SE

**Fiber development:** A general increase in fiber content in both the top and bottom portions of the control spears was observed throughout the experimental period. Although the fiber development in both top and bottom portions followed almost the same pattern, the breaking force indicating the toughness of the spears was greater in the bottom than in the top portion. In top portion of the spears AOPP at 300  $\mu$ M reduced the breaking force by 44% and in case of bottom portion by 21% (Fig. 1A and 3A). Spears treated with 100  $\mu$ M AOPP produced the

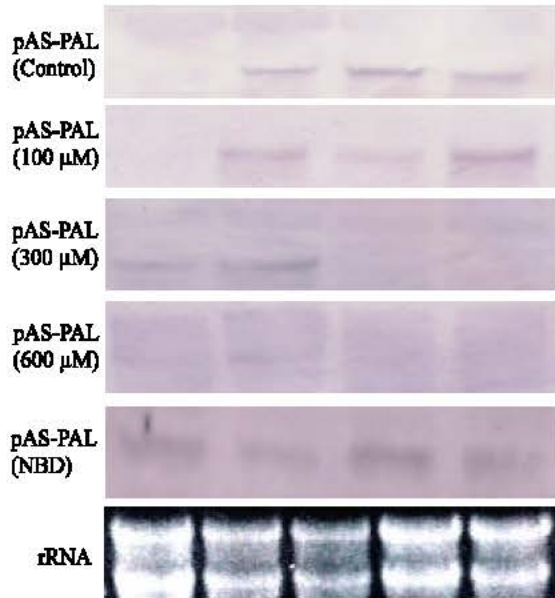


Fig. 2: Northern blot analysis showing effect of AOPP and NBD on PAL gene expression in top portion of asparagus spears. Equal loading of RNA was confirmed by staining a gel with ethidium bromide

same breaking force as control. NBD also inhibited PAL activity and resulted in almost similar fiber content like 300 μM AOPP treated spears.

### DISCUSSION

The development of PAL activity in control spears was similar in a number of respects to other wound induced tissue systems<sup>[4,15]</sup>. There was an increase in PAL activity in both top and bottom portions of the spears which continued throughout the experimental period. Although PAL activity in both top and bottom portions followed almost the same pattern the activity was higher in bottom than top portions. High PAL activity associated with bottom portion, or tissue near to cut surfaces, such as we observed, has been noted previously<sup>[6,17]</sup>. The basal tissue of the Asparagus is highly lignified and PAL activity is known to be correlated with the degree of lignification in many tissues<sup>[8,19]</sup>.

To investigate the potential role of PAL in toughening of asparagus spears, AOPP and NBD two specific inhibitors of PAL enzyme activity was applied. Both the AOPP and NBD has been well characterized in terms of its effect on PAL activity in other plant tissues<sup>[7,9]</sup>. AOPP at a concentration of 300 μM inhibited the PAL activity in both top and bottom portion of

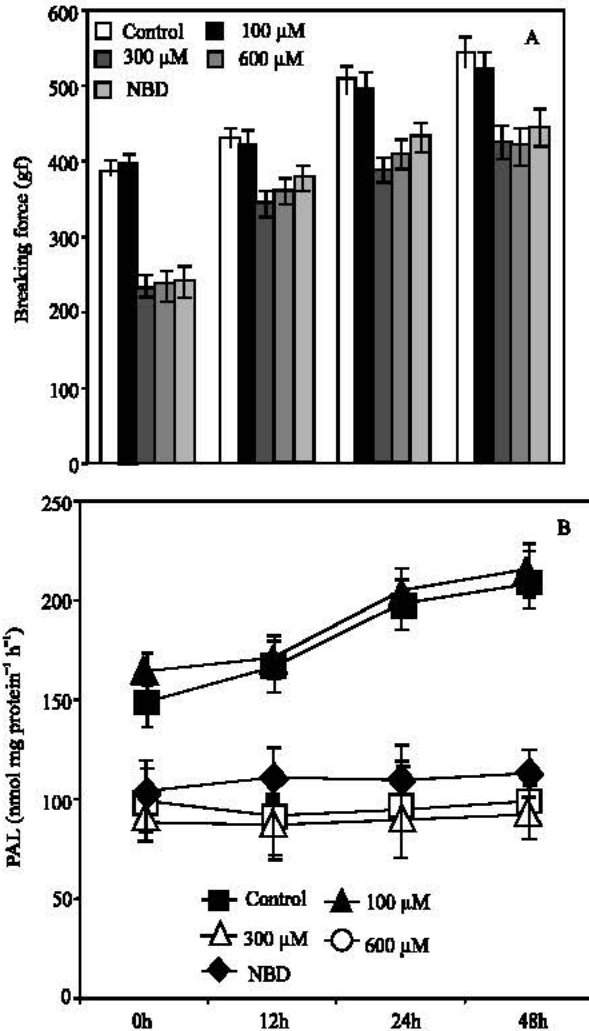


Fig. 3: Effect of AOPP and NBD on fiber content and PAL activity in bottom portion of asparagus spears. (A) Changes in breaking force. Each point represents the mean of three replicates and vertical bars show SE. (B) Changes in PAL activity. Each point represents the mean of three replicates and vertical bars show SE

spears and this was followed by reduced fiber content. We have cloned and sequenced a cDNA for PAL from asparagus spears. The 527-bp cDNA for PAL designated as pAS-PAL was a partial cDNA identical to the same region of the cDNA that encoded PAL from other plants and therefore, used as a probe for Northern blot analysis. RNA blot hybridization demonstrated that in both top and bottom portions PAL transcripts for 300 and 600 μM AOPP and NBD treated spears showed almost no or very little expression during storage. In contrast, for control and 100 μM AOPP treated spears PAL transcript

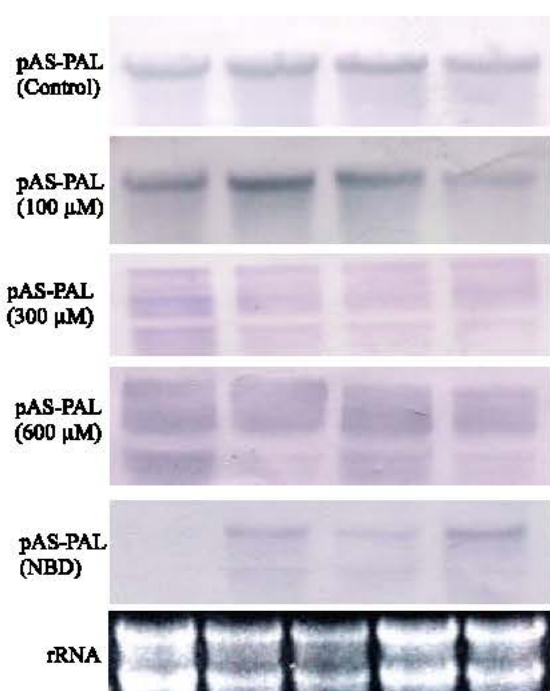


Fig. 4: Northern blot analysis showing effect of AOPP and NBD on PAL gene expression in bottom portion of asparagus spears. Equal loading of RNA was confirmed by staining a gel with ethidium bromide

accumulated just after harvest and continued up to 48 h of storage. Accumulation of PAL transcript was accompanied by increased in enzyme activity and fiber development. This increase is due to the lack of feedback regulation on PAL synthesis resulting from the very low cinnamic acid levels in the presence of inhibitors (e.g. in the presence of AOPP). Presumably NBD would have a similar effect on PAL enzyme and mRNA levels.

This suggests that PAL activity and gene expression are most influential in lignification of Asparagus spears during storage. AOPP and NBD two specific inhibitors of PAL enzyme activity, reduced PAL gene expression and decreased the fiber content of Asparagus spears, suggesting that downstream products of PAL enzyme activity may have a regulatory role in the lignification process. Therefore, safe and effective chemical means like application of AOPP and NBD could be useful to prevent or to slow the rate of toughening and increase the useable portion of stored Asparagus spears.

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