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Specific Pretreatments Reduce Curing Period of Vanilla (*Vanilla planifolia*) Beans

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With the aiming of reducing the curing period, effects of pretreatments on flavor formation in vanilla beans during accelerated curing at 38 °C for 40 days were studied. Moisture loss, change in texture, levels of flavoring compounds, and activities of relevant enzymes were compared among various pretreatments as well as the commercial sample. Use of naphthalene acetic acid (NAA; 5 mg/L) or Ethrel (1%) with blanching pretreatment resulted in 3-fold higher vanillin on the 10th day. Other flavoring compounds—vanillic acid, *p*-hydroxybenzoic acid, and *p*-hydroxybenzaldehyde—fluctuated greatly, showing no correlation with the pretreatments. Scarification of beans resulted in nearly 4- and 3.6-fold higher vanillin formations on the 10th day in NAA- and Ethrel-treated beans, respectively, as compared to control with a significant change in texture. When activities of major relevant enzymes were followed, addition of NAA or Ethrel helped to retain higher levels of cellulase throughout the curing period and higher levels of β-glucosidase on the 20th day that correlated with higher vanillin content during curing and subsequent periods. Peroxidase, being highest throughout, did not correlate with the change in levels of major flavoring compounds. The pretreatment methods of the present study may find importance for realizing higher flavor formation in a shorter period because the major quality parameters were found to be comparable to those of a commercial sample.

KEYWORDS: ***Vanilla planifolia*; accelerated curing; pretreatment; vanilla flavor; texture**

INTRODUCTION

Natural "vanilla flavor" comprises a large array of aromatic compounds formed after systematic curing of the beans of *Vanilla planifolia*. The major compound, vanillin, is the most preferred flavoring compound among the universally used aromas and has a great market potential in the food, beverage, cosmetic, and pharmaceutical industries. Sixty-five volatiles and 26 odor-active compounds are identified in the extract of cured vanilla beans (*1*); the major ones after vanillin are vanillic acid, *p*-hydroxybenzoic acid, and *p*-hydroxybenzaldehyde (**Figure 1**). In green vanilla beans, these phenolic aromatic compounds are present as their respective glucosides, the major one being glucovanillin (**Figure 1**) synthesized from phenylalanine of the shikimic acid pathway (**Figure 2**), and the curing process is meant to release the aglycones as the free aroma compounds. Curing also induces the formation of many other compounds that complement the delicate aroma of natural vanilla flavor. In fact, it is the presence of these minor compounds in large numbers that fetch a high price for natural "vanilla extract".

Botanical study of vanilla beans reveals that the flavor precursors are found in the bean interior, that is, placental region around the seeds, whereas the hydrolytic and other degenerative enzymes that are known to catalyze the reactions for the release of flavor compounds are localized mostly in the outer fruit wall (*2*). The purpose of curing is to create contact between the flavor precursors and the enzymes that catalyze the hydrolysis of precursor compounds (*3*). Curing of vanilla beans is a traditionally well-established process; it is laborious and takes 3−6 months depending on the different curing procedures adopted in different vanilla-producing regions (*3*). Despite the long time required for the curing process, the enzymatic transformation

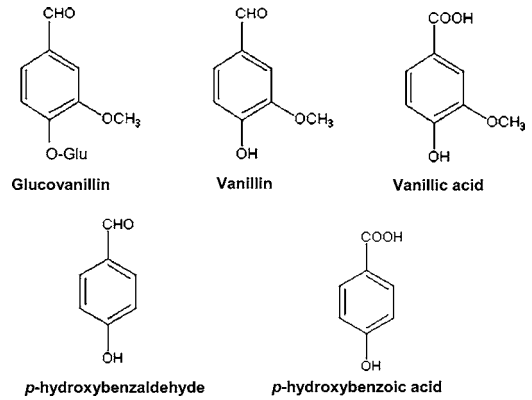

Figure 1. Chemical structures of the major flavor compounds found in cured beans of *Vanilla planifolia*.

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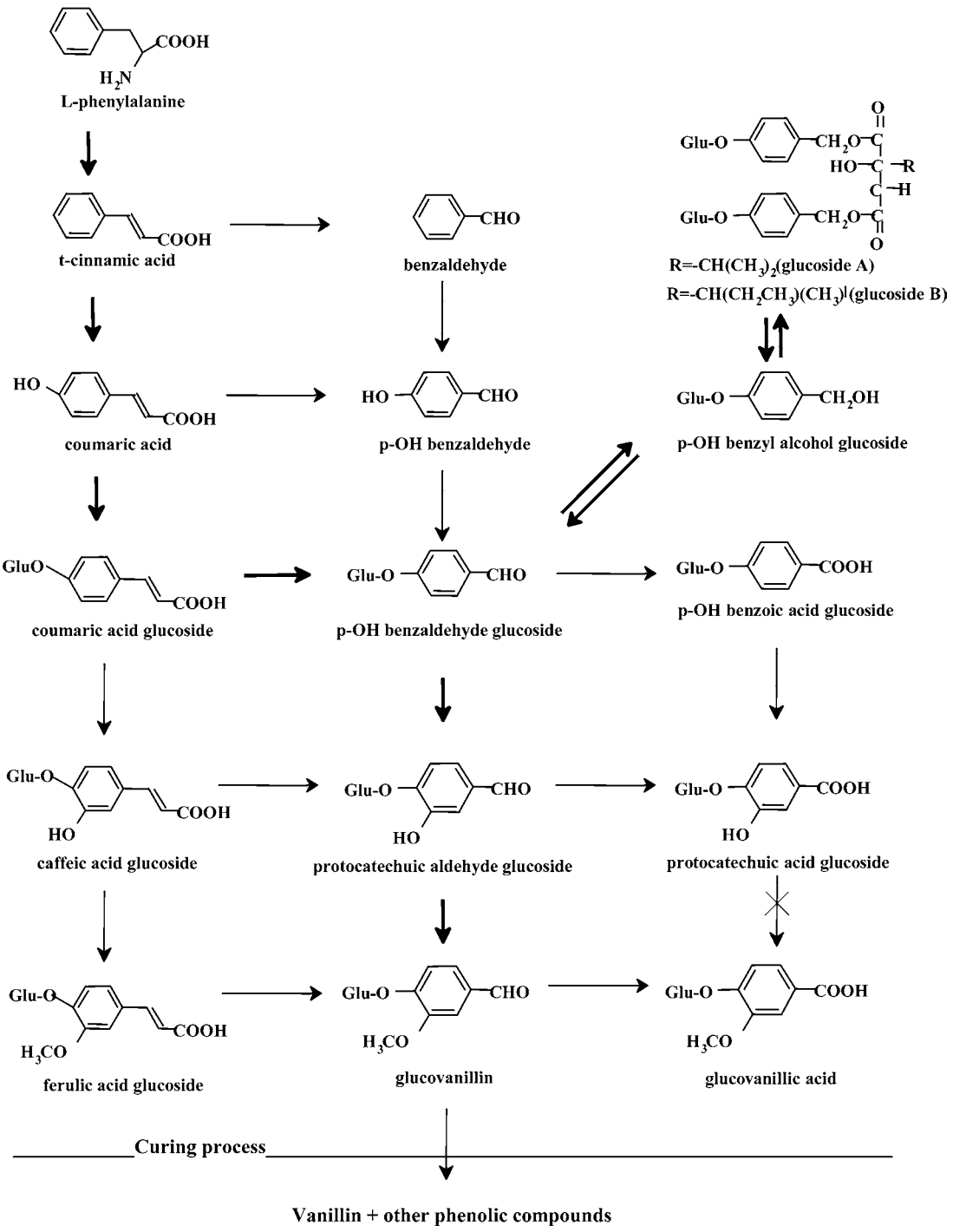

Figure 2. Shikimic acid pathway showing the formation of vanilla flavor compounds.

of the glycosides to flavoring compounds is not very efficient. Only a fraction of the vanillin is produced by systematic curing of green beans, of which a part may also be lost during exposure to sun as well as during extraction (*4*). Finally, the total flavor yield depends upon the quality of the starting material; the best quality beans range in length from 15 to 20 cm.

Conventional curing depends on weather conditions as it involves intermittent exposure to sun and sweating followed by conditioning (*5*). Therefore, it is lengthy and cumbersome, involving several months, and may often fail to completely hydrolyze glucosides, resulting in only fractions of flavor compounds (*4*).

To overcome the above problems, earlier workers used methods such as curing of the cut beans, covering beans in plastic sheets and heating them at 60 °C along with high humidity, freeze curing, treatment of green beans with various enzymes such as β-glucosidase, pectinase, and hemicellulase, hot-air drying, and solar drying (*5*, *6*). The development of vanilla flavor during these treatments is partly due to the hydrolysis of glycosylated precursors occurring in the green bean (*7*). The most important step in vanilla curing has been found to be the scalding followed by incubation at 45 °C (*8*). A similar process involving scalding in hot water of 63 °C for 2−3 min, followed by cutting the beans into pieces and incubation in an oven at 38 °C for 48 h followed by further curing in closed containers at 38 °C for 2−3 months, resulting in a pleasing aroma, was also suggested (*9*). From then onward, blanching in hot water has been an essential step traditionally performed

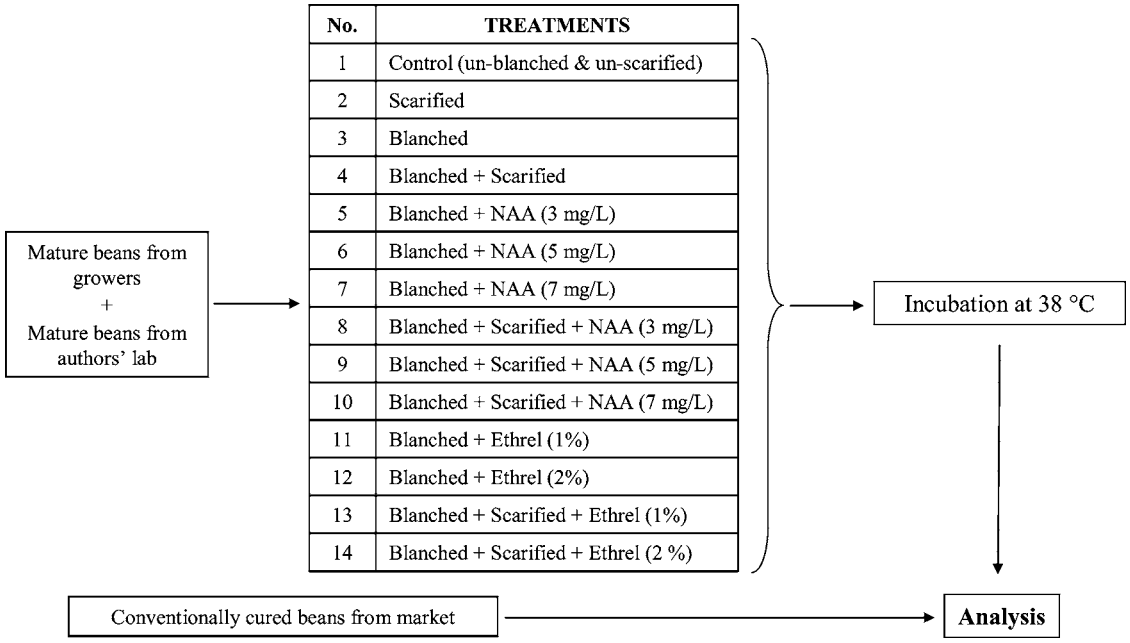

Figure 3. Experimental model adopted in the present study showing various treatments. Each treatment had at least 150 beans, whereas the market sample had 30 beans.

before curing of vanilla beans. This has been a convention for several decades in various vanilla-growing countries of the world. Mild-temperature treatment, involving sunning of the fruits, has also been a regular practice, which invariably leads to losses at each exposure.

Thus, there is a need for a process that is simple and effective. Therefore, the present study aimed at developing a novel curing process that reduces the processing time and increases the flavoring compounds in cured vanilla beans. To do this we chose to use auxin and ethrel in combination with different pretreatments. Auxins and ethrel are compounds having popularity in the area of horticulture to induce parthenocarpy and ripening/senescence, respectively. Auxins are plant growth regulators that are known to act by loosening the cell wall via enhancement of β-glucanase enzyme (*10*) as well as by acidification of cell walls (*11*), probably via inducing the secretion of hydrogen ions into and through the cell wall. Such acidification of cell wall by auxin leads to lipid breakdown, thereby increasing the extensibility of membranes and enhancing the permeability. Such effects in the present context are expected to create appropriate physicochemical conditions for the enzymes to come in contact with the substrates and act to release the flavoring compounds. A preliminary screening was done, and among the auxins, naphthalene acetic acid (NAA) was selected due to its better efficacy over others in supporting the biosynthesis of extractable phenolics (*12*, *13*). An earlier study on cell cultures of *V. planifolia* showed that NAA increased the secondary metabolism, resulting in high phenylalanine ammonia-lyase (PAL) activity and high concentration of extractable phenolics (*14*). Ethrel is widely used as a source of ethylene to induce ripening in fruits. Its involvement in the phenyl-propanoid pathway is also known and hence selected for pretreatment. The curing temperature was chosen as 38 °C, which is conducive to the activities of key enzymes involved in bioconversions of flavor precursors.

MATERIALS AND METHODS

Chemicals. Authentic vanillin, vanillic acid, *p*-hydroxybenzoic acid, and *p*-hydroxybenzaldehyde used as standards and the auxin, naphthalene acetic acid (NAA), were obtained from Sigma Chemical Co., St. Louis, MO. Ethephon (39% SL, Bayer Crop Science India Ltd., Jammu, India) served as a source for ethrel. Solvents used were HPLC-grade methanol (Ranbaxy, Gurgaon, India), triple-distilled water, and AR grade glacial acetic acid (Qualigens, Calcutta, India). Ethanol was distilled before use.

Vanilla Beans. Mature beans of vanilla ranging in length from 10 to 13 cm (second quality) were obtained from the growers in the "Western ghats" region of Karnataka, India, during the second phase of the harvesting season (early December) and transported within 24 h to the laboratory. In addition, mature beans of similar lengths were harvested from the plants grown at the authors' institute and mixed with the samples collected from the growers. For comparison with the commercial samples, conventionally cured beans from a market where the curing is done by intermittent sun-drying and a sweating process for 3−6 months (*5*) were used.

Treatments. In conventional curing, high flavor yields are achieved by way of blanching vanilla beans in hot water of 55−65 °C for 3−10 min (*5*). In the present study, a preliminary screening of different temperatures of 55, 57, 59, 61, 62, 63, 64, and 65 °C versus time ranging from 2 to 10 min was done. On the basis of this study, the beans were blanched in hot water (tap water) at a temperature of 63 °C for 3 min and spread on blotter sheets and gently pressed with fresh sheets of blotters to remove water droplets adhering to the surface of beans. The beans were allowed to stay for 30 min at room temperature (RT, 28−30 °C) until the surface of each bean appeared free from water. Scarification treatments were given by way of individually picking up the beans and creating lengthwise scarification with a brush having fine stainless steel bristles. Before the list of chemicals and their concentrations for pretreatment was compiled, a preliminary study was done wherein both NAA (3, 5, and 7 mg/L) and ethrel (1 and 2%) showed highest induction of flavor (data not shown) and hence were included for the treatment. For treatment with auxin, appropriate levels of NAA were weighed to obtain the required final concentrations (3, 5, and 7 mg/L); the compound was first dissolved in a few drops of NaOH (0.1 N) to which a required volume of lukewarm water was added and allowed to cool to RT. After blanching/nonblanching or scarification/nonscarification treatments, the beans were dipped for 5 min in NAA- or ethrel-containing water at RT. To obtain an appropriate level (1−2%) of ethrel, Ethephon was diluted with water appropriately. For the entire experiment, tap water washed untreated beans (without

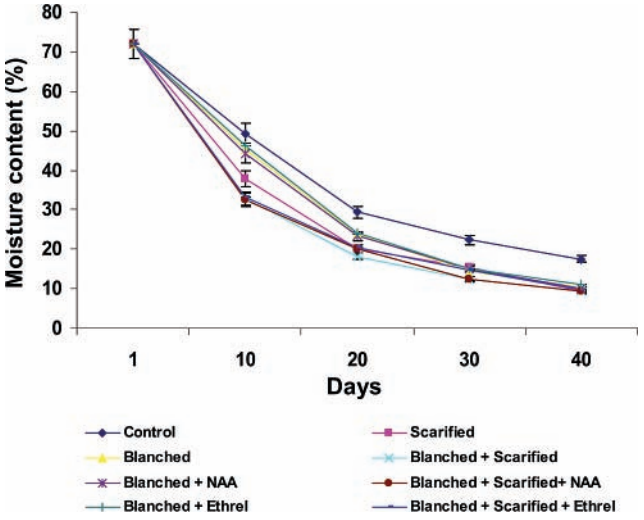

Figure 4. Moisture content at different periods of curing of vanilla beans after various pretreatments. The values presented are averages of 10 replicates ± SD.

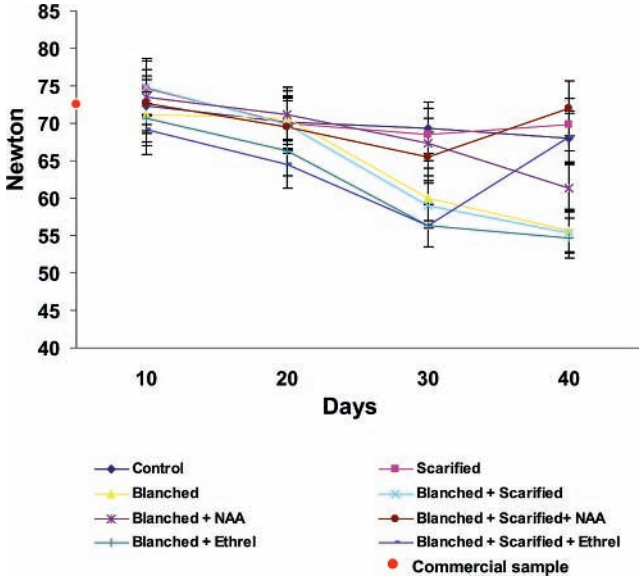

Figure 5. Texture analysis of vanilla beans during different stages of curing after various pretreatments. The values presented are averages of 10 replicates ± SD.

blanching/scarification and without NAA/ethrel treatments) served as general control; thus, all of the treatments had respective controls (**Figure 3**).

All of the samples with different treatments as well as general control were separately wrapped in double-layered butter paper and gently tied with cotton thread. Incubation of the beans was carried out at 38 °C for a period of 40 days. Because the moisture loss was very high initially, the butter wrappers were replaced with fresh ones on the 3rd, 6th, and 10th days. For each treatment, 150 beans were used. After every 10th day, 30 randomly picked beans from each treatment were used for chemical and physical analyses. After different curing periods, the beans were rebundled in fresh butter paper and allowed to condition at room temperature (28−30 °C) for 15 days, followed by storing in thermocole boxes at RT in self-sealable polythene bags.

Measurement of Moisture Loss. Beans from each treatment were separately subjected to drying in an oven at 60 °C, and the loss of water was recorded by gravimetric method throughout the experiment at required intervals.

Texture Measurement. The texture of the beans at various stages of curing was measured using an Instron 4301-UTM (Universal Texture Measuring system) by WB Shear at a speed of 100 mm/min and a load of 100 kg. Ten beans were removed from each treatment at 10 day intervals for a total curing period of 40 days and were used for the analysis. All of the results were expressed as force in newtons (N).

Flavor Analysis. *Standards.* Vanillin (1.2 g), vanillic acid (0.08 g), *p*-hydroxybenzoic acid (0.02 g), and *p*-hydroxybenzaldehyde (0.06 g) were separately weighed into a 100 mL volumetric flask and diluted to 100 mL with 95% predistilled ethanol. From these, a 10 mL aliquot was further diluted to 100 mL with 40% ethanol separately and was used as standard.

Extraction of Flavor Compounds. The extraction of flavor components from vanilla beans was done according to the method described earlier (*15*). Briefly, triplicate samples of 10 g of cured vanilla beans were finely crushed in liquid nitrogen. The extraction was done with 75 mL of 44% aqueous ethanol for 48 h at 45 °C in stoppered conical flasks. The mixture was stirred occasionally, filtered, and washed with 36% ethanol until the total volume of filtrate, along with washings, was 100 mL. An aliquot of the filtrate was taken in a syringe and passed through a membrane filter (Millipore, 0.45 μm) to remove coarse particles, and a clear aliquot was used for HPLC analyses.

Analytical High-Performance Liquid Chromatography (HPLC). The levels of flavoring compounds formed at different stages of curing were analyzed by HPLC. A Shimadzu LC 6A model (Tokyo, Japan) with a μ-Bondapack (Waters Corp., Miford, MA) C_{18} column (300 × 4.6 mm i.d., with pore size of 5 μm), an SLC-6A system controller, and a CR4A data processor was used. For calibration purposes, solutions of vanillin, vanillic acid, *p*-hydroxybenzoic acid, and *p*-hydroxybenzaldehyde at concentrations from 1 to 100 mg/L were separately prepared in 100% ethanol and injected into the HPLC system described above to build the calibration curve. Detection was done by a UV detector SPD-AV set at a sensitivity of 0.04 AUFC and a wavelength of 254 nm and a flow rate of 1 mL/min. The mobile phase used was methanol/acidified water (10:90), where 800 mL of water was acidified with 10 mL of glacial acetic acid. Samples were injected using a 20 μL sample loop injection system, and the concentrations of major flavor components were calculated by peak proportioning using the standard curves.

Extraction and Estimation of Total Protein Content. Total protein was extracted from the beans and estimated by using the macro-Kjeldahl method (*16*) because the phenolic compounds hindered the estimation by Lowry's method.

Activities of Major Enzymes. Earlier papers indicated the involvement of three major enzymes during the curing of vanilla beans: the involvement of β-glucosidase (β-GLUC) for catalyzing the conversion of glucovanillin and other glycosides to vanillin and respective flavor compounds, cellulase (CSE) for cell-wall degradation assisting the permeabilization of β-GLUC from the surface of the beans to the center, and peroxidase (POD) in various bioconversions of phenyl-propanoid compounds (*5*). Therefore, the activities of these three enzymes were followed throughout the curing period after different treatments.

Extraction of Enzymes and Assay. *Extraction.* The extraction involved chopping of beans into 1 cm pieces, of which 5 g was extracted at 4 °C with 20 mL of respective buffers and centrifuged twice at 5000*g* for 15 min, and the supernatant was used as the enzyme source.

β-Glucosidase. The buffer used was 0.1 M sodium citrate (pH 5). The activity of β-GLUC was determined at 30 °C in fresh extraction buffer (pH 5) using *p*-nitrophenol glucopyranoside as the substrate. The *p*-nitrophenol released after the glucose is hydrolyzed by the enzyme is measured spectrophotometrically. The reaction mixture contained 100 μL of 0.1 M sodium citrate buffer (pH 5), 100 μL of enzyme extract, and 100 μL of 0.0055 M *p*-nitrophenol β-D-glucopyranoside (*17*). The hydrolysis of 1 μmol of substrate/min was recorded as 1 unit of activity and quantified on the basis of $\epsilon = 18500/\text{mol cm}^{-1}$.

Cellulase. CSE activity was determined by measuring the reducing groups released from carboxymethyl cellulose (CMC, Sigma) by following the method explained elsewhere (*18*). The reaction mixture contained 0.25 mL of crude enzyme, 0.5 mL of 0.1% (w/v) CMC, and 0.25 mL of sodium citrate buffer (pH 5), incubated at 37 °C for 1 h. One unit is defined as the amount of enzyme that catalyzed the formation of 1 μM reducing group/min.

Peroxidase. POD was extracted in sodium phosphate buffer (pH 6) at 4 °C, and the activity was determined following the procedure explained elsewhere (*19*). Briefly, 1 mL of assay mixture was prepared,

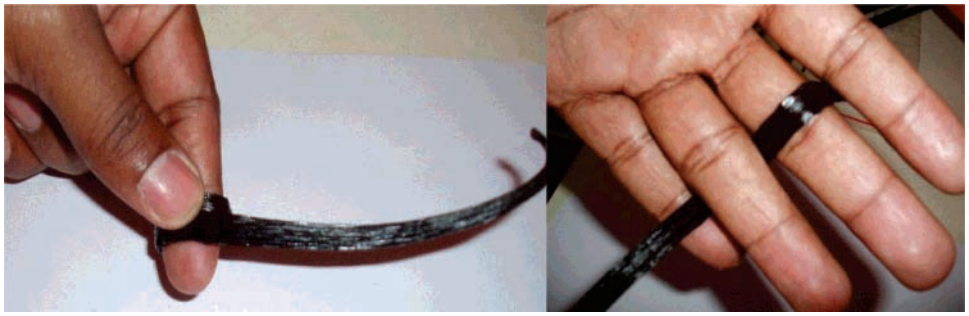

Figure 6. Vanilla bean cured for 10 days after pretreatment with NAA during blanching pretreatement. The figure shows the flexibility of the bean stored for 1 year at room temperature in air-tight pouches.

which consisted of 100 μL of 1% H_2O_2, 100 μL of 0.25% *o*-dianisidine dihydrochloride, 10 μL of enzyme extract, and 790 μL of sodium phosphate buffer (pH 6). The change in absorbance at 460 nm per minute (dA min^{-1}) at 27 °C was recorded using a kinetic program in a UV−visible spectrophotometer (Shimadzu UV-160A). Activity was quantified on the basis of a standard curve, using the same substrate, of horseradish POD enzyme obtained from ICN Biochemicals. One unit of enzyme activity refers to the rate of change of absorbance by 1 unit/min.

Statistical Analyses. Each treatment had at least 150 beans, and 30 beans were randomly picked, at a known point of time, for physical and chemical analyses. The entire experiment was repeated in the subsequent year. Student's *t* test has been used to compare the mean values, and the tests were considered to be statistically significant at $p < 0.05$. The data were analyzed by one-way analysis of variance (ANOVA) using Microsoft Excel XP (Microsoft Corp., Redmond, WA), and post-hoc mean separations were performed by Duncan's multiple-range test at $p \leq 0.05$ (*20*).

RESULTS

Changes in Physical Parameters. The pattern of water loss in vanilla beans during the curing period of 40 days at 38 °C is shown in **Figure 4**. The initial water content of >70% was drastically reduced, in most of the treatments, to around 10% (w/w of the beans) by the 40th day except in the control. The water loss was higher in treated beans than in the control, and greater in scarification treatments. The conventionally cured market samples were also subjected to this test and found to contain 25% moisture. The analysis of texture (**Figure 5**) showed progressively low values, indicating increase in softness until the end of the curing period in most of the treatments. However, at the end of the curing period the control and scarified samples showed higher resistance due to greater hardness than in other treatments. The conventionally cured commercial sample showed texture comparable to that of the 10 days cured samples of the present study, indicating that 10th day is the right time to terminate the incubation at 38 °C. The NAA-pretreated beans cured for 10 days and conditioned at RT retained their flexibility even after 1 year (**Figure 6**). Thus, both texture and moisture levels of 10 day cured and conditioned at RT beans are comparable to those of the commercial sample in almost all of the treated beans except for unblanched control.

Formation of Flavor Compounds. HPLC profiles of standard flavoring compounds and those of variously cured beans are shown in **Figure 7**. Vanillin, the major flavoring compound, was formed to various degrees with respect to different treatments (**Table 1A**). After 10 days of incubation, the control (unblanched and unscarified) showed a substantial quantity of vanillin formed, which declined steadily up to the 30th day followed by a negligible improvement on the 40th day. Scarification of beans enhanced vanillin content by nearly 40% on the 10th day, which decreased further during the entire curing period of 40 days, showing a 15−25% increase over the respective control, that is, unscarified beans (**Table 1A**).

Blanching, the conventional pretreatment, showed an increase of nearly 1.5-fold (150% increase) in vanillin content when compared to unblanched control after 10 days of curing. A very significant increase of vanillin content by nearly 2-fold was evinced when blanching was combined with scarification treatment compared to blanching alone. A similar 2-fold increase in vanillin was observed when NAA (5 mg/L) treatment followed the blanching treatment when compared to the respective control, that is, blanching alone. Although all of the treatments with NAA resulted in significant increase in the turnover of vanillin and other flavoring compounds, the best concentration was 5 mg/L. When blanching and scarification were combined with NAA treatment (5 mg/L), there was a very high increase in vanillin formation, accounting for a nearly 4-fold increase as compared to untreated control and 2.6-fold higher than blanched control on the 10th day of curing. In this treatment, the increase was 4-fold over the control on the 10th, 20th, and 40th days compared to control (untreated). Other concentrations of NAA, that is, 3 and 7 mg/L, were less efficient than 5 mg/L. Between the two ethrel treatments, use of 1% ethrel after blanching and scarification showed significantly higher vanillin content on 10th day than the respective control, that is, blanching and scarification (**Table 1A**).

With regard to the other major flavoring compounds, vanillic acid concentration showed a significant increase in control beans after 10 days of incubation and for blanched + scarified + NAA (5 mg/L) treated beans 20 days after incubation (**Table 1B**). Concentration of *p*-hydroxybenzoic acid was higher in control on the 10th and 20th days of curing than in the pretreated ones, which had a fall after the 20th day (**Table 1C**). There was a significant increase (3-fold) in *p*-hydroxybenzaldehyde concentration when beans were blanched and incubated for 20 days. Blanching followed by scarification and NAA (5 mg/L) treatment showed an increase of 2.4-fold compared to control on the 30th day (**Table 1D**).

Protein Content. Total protein content estimated on the basis of nitrogen analysis showed the presence of 5.64% protein on a 25% moisture basis. This value remained constant without any significant change throughout the curing process (**Figure 8**).

Enzyme Activities. *β-Glucosidase.* In general, incubation at 38 °C for 10 days showed a significant increase in the activity of β-GLUC compared to the initial activity in green beans (172.3 units/g of tissue at a 25% moisture level). Blanched beans exhibited a higher activity on the 10th day, whereas the activity declined significantly on the 20th and 30th days of curing (**Table 2A**). Beans blanched, scarified, and treated with NAA (5 mg/L) as well as those blanched, scarified, and treated with 1%

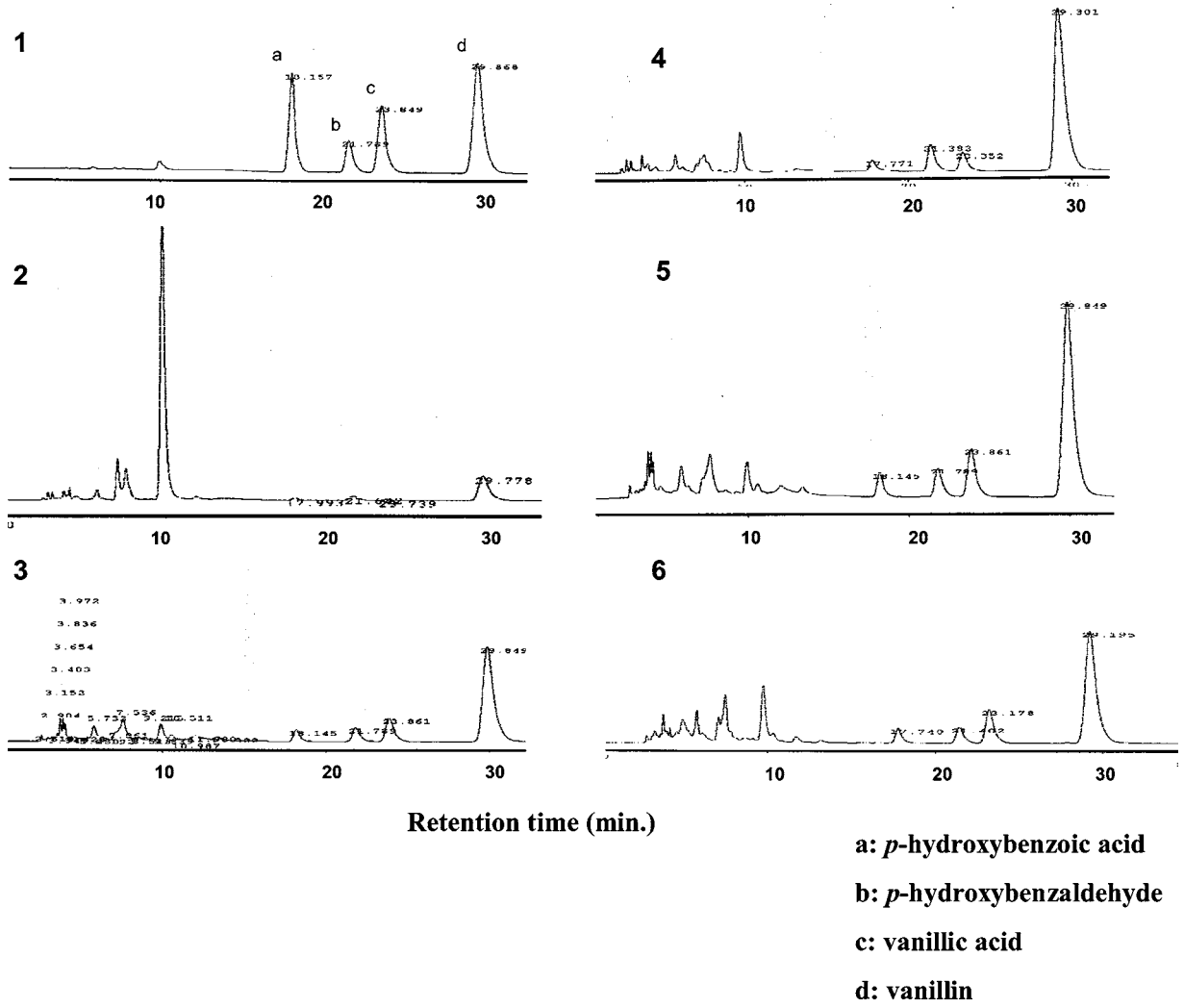

Figure 7. HPLC profiles of standard compounds (1), green bean (2), blanched bean (3), ethrel-pretreated bean (blanched, scarified, and treated with 1% ethrel) (4), NAA-pretreated bean (blanched, scarified, and treated with 5 mg/L of NAA) (5), and commercial bean sample (6) showing elution time for major vanilla flavor compounds detected at 254 nm. HPLC profiles 3–5 are of beans incubated for 10 days at 38 °C.

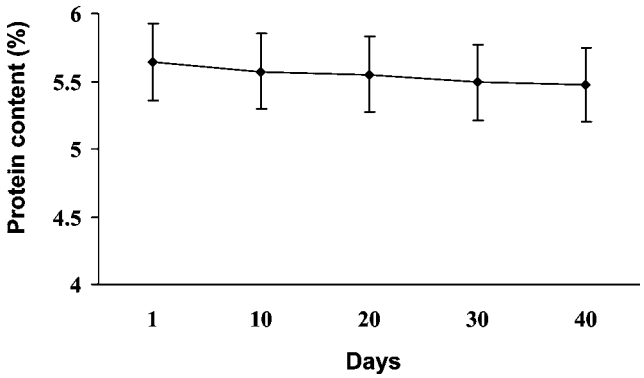

Figure 8. Total protein content of vanilla beans (at 25% moisture level) at different incubation periods as estimated by using the macro-Kjeldahl method.

ethrel showed an increased activity on the 20th day. The enzyme activity declined upon further incubation.

Cellulase. All of the pretreatments including blanching showed a significant increase in the CSE activity compared to initial activity (0.74 unit/g of tissue at a 25% moisture level) in green beans. Although there was a higher activity on the 20th day in all of the treatments, a sudden fall was noticed upon further incubation (**Table 2B**).

Peroxidase. An initial POD activity of 1125.3 units/g of tissue (at a 25% moisture level) was found in the green beans, which increased upon scarification treatment on the 10th day and increased further on the 20th day. Blanching generally retarded the activity compared to the unblanched control, although uniformity in the activity was found in most of the treatments throughout the curing period (**Table 2C**).

DISCUSSION

Traditionally, the appearance, flexibility, and size characteristics of vanilla beans have been of importance because there is a fairly close relationship between any these two factors and the aroma/flavor quality. The moisture content is one of several parameters that are important for bean quality. It is, therefore, very important to realize that moisture content is interdependent on other quality parameters and cannot be considered, by itself, as an index of quality. The presence of appropriate water content ranging from 25 to 30% has been noted by earlier workers to result in the desirable texture. The present study has clearly shown that the use of NAA or ethrel coupled with bean scarification more quickly reduced the moisture content than a similar set of conditions with blanching treatment (**Figure 4**). Beans used for extraction have low moisture content, whereas gourmet beans have a higher moisture content (*2*). The moisture content is a major factor in the preservation of cured vanilla

Table 1. Concentrations of Vanillin, Vanillic Acid, *p*-Hydroxybenzoic Acid, and *p*-Hydroxybenzaldehyde in Control and Pretreated Vanilla Beans Analyzed during Different Stages of Curing[a]

S1	treatment	10 DAC	20 DAC	30 DAC	40 DAC
	(**A**) Vanillin (Percent per Gram of Bean Weight[b])				
1	control	0.546 de	0.518 de	0.421 de	0.463 d
2	scarified	0.759 d	0.655 d	0.486 de	0.586 d
3	blanched	**0.821 d**	1.362 bc	1.151 c	1.287 bc
4	blanched + scarified	**1.583 bc**	1.422 bc	1.584 b	1.988 a
5	blanched + NAA (3 mg/L)	1.29 c	1.671 b	1.235 c	1.612 b
6	blanched + NAA (5 mg/L)	**1.692 ab**	2.137 a	2.071 a	2.022 a
7	blanched + NAA (7 mg/L)	1.41 bc	1.823 ab	1.461 bc	1.822 ab
8	blanched + scarified + NAA (3 mg/L)	1.301 c	1.265 c	0.912 cd	1.218 bc
9	blanched + scarified + NAA (5 mg/L)	**2.174 a**	2.058 a	1.513 a	1.945 a
10	blanched + scarified + NAA (7 mg/L)	1.541 bc	1.412 bc	1.216 c	1.564 b
11	blanched + ethrel (1%)	1.612 ab	1.911 ab	1.641 b	1.844 ab
12	blanched + ethrel (2%)	1.411 bc	1.713 b	1.414 bc	1.623 b
13	blanched + scarified + ethrel (1%)	**1.983 ab**	1.962 ab	1.72 ab	1.956 a
14	blanched + scarified + ethrel (2%)	1.562 bc	1.642 b	1.513 b	1.428 b
	(**B**) Vanillic Acid (Percent per Gram of Bean Weight[b])				
1	control	**0.009 a**	0.002 d	**0.013 a**	0.001 cd
2	scarified	0.005 bc	0.001 de	0.004 cd	0.002 cd
3	blanched	0.003 bc	0.003 cd	0.003 cd	0.008 ab
4	blanched + scarified	0.003 bc	0.001 de	0.004 cd	0.009 a
5	blanched + NAA (3 mg/L)	0.001 de	0.002 d	0 e	0 d
6	blanched + NAA (5 mg/L)	0.002 d	0.003 cd	0.002 d	0.002 cd
7	blanched + NAA (7 mg/L)	0 e	0.001 de	0.001 de	0 d
8	blanched + scarified + NAA (3 mg/L)	0.003 cd	0.004 bc	0.006 c	0 d
9	blanched + scarified + NAA (5 mg/L)	0.005 bc	**0.007 a**	0.012 ab	0 d
10	blanched + scarified + NAA (7 mg/L)	0.004 c	0.005 b	0.005 c	0 d
11	blanched + ethrel (1%)	0.004 c	0.005 b	0.009 b	0.004 bc
12	blanched + ethrel (2%)	0.002 d	0.003 cd	0.007 bc	0.001 cd
13	blanched + scarified + ethrel (1%)	0.001 de	0.004 bc	0.007 bc	0.007 ab
14	blanched + scarified + ethrel (2%)	0 e	0.003 cd	0.005 c	0.006 b
	(**C**) *p*-Hydroxybenzoic Acid (Percent per Gram of Bean Weight[b])				
1	control	**0.003 a**	**0.006 a**	0.003 b	0.001 b
2	scarified	0.002 b	0.002 c	0 e	0.002 a
3	blanched	0.001 c	0.003 bc	0.002 c	0.002 a
4	blanched + scarified	0.001 c	0.001 d	0.001 d	0.002 a
5	blanched + NAA (3 mg/L)	0 d	0 e	0.001 d	0.001 b
6	blanched + NAA (5 mg/L)	0.001 c	0.001 d	0.001 d	0.001 b
7	blanched + NAA (7 mg/L)	0 d	0 e	0 e	0 c
8	blanched + scarified + NAA (3 mg/L)	0 d	0 e	0.002 c	0.001 b
9	blanched + scarified + NAA (5 mg/L)	0.001 c	0.001 d	0.004 a	0.002 a
10	blanched + scarified + NAA (7 mg/L)	0 d	0 e	0.003 b	0.001 b
11	blanched + ethrel (1%)	0.002 b	0 e	0.003 b	0 c
12	blanched + ethrel (2%)	0.001 c	0 e	0.002 c	0 c
13	blanched + scarified + ethrel (1%)	0.001 c	0.001 d	0.002 c	0.002 a
14	blanched + scarified + ethrel (2%)	0 d	0.001 d	0.001 d	0.001 b
	(**D**) *p*-Hydroxybenzaldehyde (Percent per Gram of Bean Weight[b])				
1	control	0.002 d	0.004 d	0.007 c	0.002 c
2	scarified	0.008 a	0.003 d	0.002 de	0.002 c
3	blanched	0.005 bc	**0.013 a**	0.002 de	0.005 a
4	blanched + scarified	0.003 cd	0.003 de	0.003 c	**0.005 a**
5	blanched + NAA (3 mg/L)	0.002 d	0.001 e	0 e	0.001 cd
6	blanched + NAA (5 mg/L)	0.004 c	0.003 de	0.002 de	0.002 de
7	blanched + NAA (7 mg/L)	0.001 de	0.002 de	0.001 de	0 de
8	blanched + scarified + NAA (3 mg/L)	0 e	0.004 d	0.008 c	0 e
9	blanched + scarified + NAA (5 mg/L)	0.001 de	0.009 bc	**0.017 a**	0.002 de
10	blanched + scarified + NAA (7 mg/L)	0 e	0.005 cd	0.006 cd	0.001 de
11	blanched + ethrel (1%)	0.001 de	0.002 de	0.009 bc	0.003 b
12	blanched + ethrel (2%)	0 e	0.001 e	0.001 de	0.001 cd
13	blanched + scarified + ethrel (1%)	0.003 cd	0.007 c	0.004 d	0.003 b
14	blanched + scarified + ethrel (2%)	0.002 d	0.004 d	0.002 de	0.001 cd

[a] Data are the mean value of three replicates and from two different seasons. Data followed by different letters within each column are significantly different according to Duncan's multiple-range test at $p < 0.05$. DAC, days after curing. [b] At 25% moisture level.

beans because low moisture content is essential to prevent microbial growth. Also, the water content of properly cured beans must be sufficiently low to prevent the growth and activity of microorganisms, because low water, in combination with high phenolic content, offers protection against spoilage in cured beans. Our observation of NAA/ethrel-treated 10-day-cured beans stored at RT for 1 year did not show spoilage due to any fungal/microbial infestation (**Figure 6**).

The present study has documented the texture measurement of vanilla beans for the first time. Because the desired moisture content in vanilla beans and the texture comparable to that of commercial samples were achievable in a period of 10 days,

Table 2. Activities of β-Glucosidase, Cellulase, and Peroxidase in Control and Pretreated Vanilla Beans Analyzed during Different Stages of Curing[a]

S1	treatment	0 day	10 DAC	20 DAC	30 DAC	40 DAC
		(**A**) β-Glucosidase Activity (Units per Gram of Tissue[b])				
1	control	172.3 ± 9.3	188.094 bc	311.306 bc	141.133 cd	119.846 cd
2	scarified		187.013 bc	315.898 bc	208.593 bc	204.085 bc
3	blanched		**257.263 a**	**243.352 cd**	**126.931 d**	201.480 bc
4	blanched + scarified		213.74 b	358.14 bc	318.660 a	246.639 b
5	blanched + NAA (3 mg/L)		105.90 d	174.9 d	101.21 d	108.55 d
6	blanched + NAA (5 mg/L)		211.876 b	349.875 bc	134.032 cd	217.112 bc
7	blanched + NAA (7 mg/L)		110.2 d	190.2 d	120.4 d	126.2 cd
8	blanched + scarified + NAA (3 mg/L)		102.69 de	243.35 cd	100.24 d	106.22 d
9	blanched + scarified + NAA (5 mg/L)		205.39 b	**486.703 a**	192.616 c	183.242 c
10	blanched + scarified + NAA (7 mg/L)		114.24 d	200.26 d	108.22 d	123.21 cd
11	blanched + ethrel (1%)		178.365 bc	417.830 ab	227.234 bc	274.429 ab
12	blanched + ethrel (2%)		156.21 c	316.24 bc	196.21 c	246.22 b
13	blanched + scarified + ethrel (1%)		193.499 bc	**470.174a**	265.402 ab	308.299 a
14	blanched + scarified + ethrel (2%)		176.22 bc	373.41 b	246.24 b	278.42 ab
		(**B**) Cellulase Activity (Units per Gram of Tissue[b])				
1	control	0.74 ± 0.12	0.972 cd	3.007 ab	0.589 d	0.864 ab
2	scarified		1.238 cd	2.103 bc	0.437 de	0.623 c
3	blanched		2.637 ab	0.914 d	0.598 d	0.716 bc
4	blanched + scarified		2.684 ab	2.093 bc	1.567 a	0.976 a
5	blanched + NAA (3 mg/L)		1.342 c	1.682 c	0.304 e	0.464 d
6	blanched + NAA (5 mg/L)		2.695 ab	3.363 a	0.608 d	0.929 ab
7	blanched + NAA (7 mg/L)		1.462 c	1.841 c	0.421 de	0.562 cd
8	blanched + scarified + NAA (3 mg/L)		1.324 c	1.415 cd	0.308 e	0.446 d
9	blanched + scarified + NAA (5 mg/L)		2.648 ab	2.83 b	0.617 d	0.892ab
10	blanched + scarified + NAA (7 mg/L)		1.562 c	1.678 c	0.546 d	0.729 bc
11	blanched + ethrel (1%)		2.96 a	3.272 ab	0.665 d	0.819 b
12	blanched + ethrel (2%)		2.746 ab	2.944 ab	0.428 de	0.662 c
13	blanched + scarified + ethrel (1%)		2.81 a	3.484 a	0.592 d	0.94 a
14	blanched + scarified + ethrel (2%)		2.442 ab	2.991 ab	0.322 e	0.774 b
		(**C**) Peroxidase Activity (Units per Gram of Tissue[b])				
1	control	1125.3 ± 29.4	1492.24 ab	1697.55 bc	1524.67 a	1517.48 a
2	scarified		**1749.64 a**	**2541.84 a**	513.78 d	592.27 d
3	blanched		1278.70 bc	2104.65 ab	1109.86 b	308.36 e
4	blanched + scarified		995.38 c	1038.63 cd	539.65 d	1010.81 ab
5	blanched + NAA (3 mg/L)		589.24 de	554.32 de	398.45 de	347.47 de
6	blanched + NAA (5 mg/L)		1178.48 bc	1108.65 cd	796.91 cd	694.94 cd
7	blanched + NAA (7 mg/L)		624.42 d	739.10 d	531.27 d	463.29 de
8	blanched + scarified + NAA (3 mg/L)		634.45 d	559.45 de	332.87 e	352.31 de
9	blanched + scarified + NAA (5 mg/L)		1268.9 bc	1118.90 cd	665.74 cd	704.62 cd
10	blanched + scarified + NAA (7 mg/L)		845.9 cd	745.93 d	443.82 de	469.74 de
11	blanched + ethrel (1%)		1359.32 b	1182.91 cd	787.01 cd	753.05 cd
12	blanched + ethrel (2%)		906.21 cd	801.46 d	524.67 d	502.03 d
13	blanched + scarified + ethrel (1%)		1404.53 b	1277.64 c	816.46 c	779.69 cd
14	blanched + scarified + ethrel (3%)		936.35 cd	851.76 d	544.3 d	519.79 d

[a] Data are the mean value of three replicates and from two different seasons. Data followed by different letters within each column are significantly different according to Duncan's multiple-range rest at $p < 0.05$. DAC, days after curing. [b] At 25% moisture level.

the curing conditions (time and temperature) selected in the present study appear to be appropriate to accomplish faster curing. Supporting this are the data of the HPLC profiles of flavors, which are also comparable to those of the commercial sample (**Figure 7**). Thus, the present study has clearly demonstrated the possibility of reducing the curing period of vanilla beans to as short as 10 days with an almost similar array of flavoring compounds and texture compared to the conventionally cured ones. However, for the formation of age-related compounds, which takes place during the conditioning of the beans after the curing period, the cured beans need to be stored appropriately. Therefore, the cured beans that result after NAA/ethrel pretreatment also need to be conditioned as per the traditional methods.

Although several papers have indicated the absence of vanillin in the green beans, the results of the present study showed that some amount of vanillin and negligible amounts of other flavoring compounds were already formed in green beans before the onset of the curing process (**Figure 7**). It appears that the glucosyl form of vanillin is largely predominant in green beans, but it does not exclude the presence of a fraction in free form (*21*). This indicates that the glucolytic and other hydrolytic enzymes within the vanilla beans are activated immediately after harvest. Blanching, a traditional curing process (Bourbon method), substantially enhanced the flavor formation from the respective glucosides, as compared to untreated beans, despite the well-documented fact that the specific β-GLUC is arrested after blanching and missing during curing (*22*). An extensive study of the thermal sensitivity of β-GLUC showed that the activity of the enzyme was lost within 24 h after blanching (*22*). However, in the present study, a much higher activity than reported elsewhere was observed throughout the curing period, where most of the treatments significantly enhanced the enzyme activity on the 20th day. Although direct correlation could not be made between the enzyme levels and the quantity of flavors formed at a given period of treatment, one can reason that such a fluctuation in turn-over of each compound is due to either interconversions of various precursor compounds or variations in reaction kinetics. The biosynthetic pathway of vanillin and other flavor-related compounds is shown in **Figure 2**. The

formation of vanillin precursor, glucovanillin, originates from glucosides of ferulic acid or protocatechuic aldehyde, both compounds being derived from the coumaric acid glucoside of phenyl-propanoid pathway (**Figure 2**). In the study of the biosynthesis of vanillin in vanilla beans, it was found that ferulic acid was incorporated to a greater extent into vanillin than vanillic acid, indicating that ferulic acid is β-oxidized to vanillyl-Co-A, which can be either reduced to vanillin or deacetylated to vanillic acid (*23*). Thus, the native β-glucosidase plays a crucial role in the conversion of not only the glucovanillin into vanillin but also the other precursors to glucovanillin. This probably could be the reason for fluctuations in the levels of flavoring compounds during the curing period and hence is not strictly corroborative with the enzyme levels. The limiting factor in glycoside hydrolysis is more likely to be the cellular compartmentation than the enzyme activity level. Glucoside hydrolysis is complete if the treatment allows a total decomparmentation and if sufficient residual glucosidase activity can continue (*21*). Native peroxidase, which was found to be thermostable and persisted with high activity throughout the curing process, might be responsible for the oxidation of vanillin to produce quinine bodies having a different aroma from vanillin and, thus, be partially responsible for the overall loss of vanilla aroma and fluctuation in vanillin content. Also, the continuous escape of flavoring molecules into the air leading to erroneous quantifications cannot be ignored. This is also indicative of substantial flavor losses occurring in conventional curing, where routine sunning is involved. The pretreatments appear to enhance the flavor formation via up-regulation of the β-GLUC and CSE.

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LITERATURE CITED

(1) Perez-Silva, A.; Odoux, E.; Brat, P.; Ribeyre, F.; Rodriguez-Jimenes, G.; Robles-Olvera, V.; Garcia-Alvarado, M. A.; Gunata, Z. GC-MS and GC-olfactometry analysis of aroma compounds in a representative organic aroma extract from cured vanilla (*Vanilla planifolia* G. Jackson) beans. *Food Chem.* **2006**, *99*, 728−735.

(2) Havkin-Frenkel, D.; French, J. C.; Pak, F. E.; Frenkel, C. Inside vanilla: *Vanilla planifolia*'s botany, curing options and future market prospects. *Perfum. Flavor.* **2005**, *30*, 36−55.

(3) Havkin-Frenkel, D.; French, J. C.; Graft, N. M.; Pak, F. E.; Frenkel, C.; Joel, D. M. Interrelation of curing and botany in vanilla (*Vanilla planifolia*) bean. *Acta Hortic.* **2004**, *629*, 93−101.

(4) Frenkel, C.; Havkin-Frenkel, D. The physics and chemistry of vanillin. *Perfum. Flavor.* **2006**, *31*, 28−44.

(5) Dignum, M. J. W.; Kerler, J.; Verpoorte, R. Vanilla production: technological, chemical, and biosynthetic aspects. *Food Rev. Int.* **2001**, *17*, 199−219.

(6) Ruiz-Teran, F.; Perez-Amador, I.; Lopez-Munguia, A. Enzymatic extraction and transformation of glucovanillin to vanillin from vanilla green pods. *J. Agric. Food Chem.* **2001**, *49*, 5207−5209.

(7) Arana, F. E. Action of a β-glucosidase in the curing of vanilla. *Food Res. Int.* **1943**, *8*, 343−351.

(8) Jones, M. A.; Vincente, G. C. Criteria for testing vanilla in relation to killing and curing methods. *J. Agric. Res.* **1949**, *78*, 425−434.

(9) Broderick, J. J. The science of vanilla curing. *J. Food Technol.* **1956**, *10*, 184.

(10) Felix, G.; Meins, F. Purification, immunoassay and characterization of an abundant cytokinin-regulated polypeptide in cultured tobacco tissues. *Planta* **1985**, *164*, 423−428.

(11) Schubert, S.; Matzke, H. Influence of phytohormones and other effectors on proton extrusion by isolated protoplasts from rape leaves. *Plant Physiol.* **1985**, *64*, 285−289.

(12) Funk, C.; Brodelius, P. E. Phenylpropanoid metabolism in suspension cultures of *Vanilla planifolia* Andr. II Effects of precursor feeding and metabolic inhibitors. *Plant Physiol.* **1990**, *94*, 95−101.

(13) Funk, C.; Brodelius, P. E. Phenylpropanoid metabolism in suspension cultures of *Vanilla planifolia* Andr. III Conversion of 4-methoxycinnamic acids into 4-hydroxybenzoic acids. *Plant Physiol.* **1990**, *94*, 102−108.

(14) Funk, C.; Brodelius, P. E. Influence of growth regulators and an elicitor on phenyl propanoid metabolism in suspension cultures of *Vanilla planifolia*. *Phytochemistry* **1990**, *29*, 845−848.

(15) Ranadive, A. S. Vanillin and related flavour compounds in vanilla extracts made from beans of various global origins. *J. Agric. Food Chem.* **1992**, *40*, 1922−1924.

(16) AOAC. *Official Methods of Analysis*, 17th ed.; Association of Official Analytical Chemists: Washington, DC, 2000; no. 984.13.

(17) Spagna, G.; Barbagallo, N. R.; Palmeri, R.; Restuccia, C.; Giudici, M. Properties of endogenous β-glucosidase of a *Saccharomyces cerevisiae* strain isolated from Sicilian musts and wines. *Enzyme Microb. Technol.* **2002**, *31*, 1030−1035.

(18) Priya-Sethu, K. M.; Prabha, T. N.; Tharanathan, R. N. Post-harvest biochemical changes associated with the softening phenomenon in *Capsicum annuum* fruits. *Phytochemistry* **1996**, *42*, 961−966.

(19) Agostini, E.; Milard de Forchetti, S.; Tigier, H. A. Production of peroxidases by hairy roots of *Brassica napus*. *Plant Cell Tiss. Org. Cult.* **1997**, *47*, 177−182.

(20) Harter, L. N. Critical values for Duncan's new multiple range test. *Biometrics* **1960**, *16*, 671−685.

(21) Odoux, E. Glucosylated aroma precursors and glucosidase(s) in vanilla bean (*Vanilla planifolia* G. Jackson). *Fruits* **2006**, *61*, 171−184.

(22) Dignum, M. J. W.; Joseph, K.; Verpoorte, R. Vanilla curing under lab conditions. *Food Chem.* **2002**, *79*, 165−171.

(23) Zenk, M. H. Biosynthese von vanillin in *Vanilla planifolia* Andr. *Pflanzen. Physiol.* **1965**, *53*, 404.

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