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Biotic elicitors enhance flavour compounds during accelerated curing of vanilla beans

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ABSTRACT

Food-grade elicitors, selected after an initial screening, were used in combination with pre-treatments for the accelerated curing of beans. When acetone dried red beet elicitor – a rich source of peroxidase (98,500 U g^{-1} dry weight) was used, 2.65% vanillin was formed in 10 days, which was 1.7-fold higher than in control beans (blanched + scarified) of this study and 3.23-fold higher than the conventional curing (0.82%). HPLC analysis of elicitor-treated samples showed the formation of almost all the major compounds found in the conventionally cured beans (cured for 3–6 months) with better sensorial properties. These observations appear useful for developing a rapid process for the curing of vanilla beans.

1. Introduction

Natural vanilla flavour, obtained from cured beans of *Vanilla planifolia* forms the highest-priced flavour ingredient in food (60%), cosmetics (33%) and aromatherapy (7%) (Priefert, Rabenhorst, & Steinbiichel, 2001). Characteristic vanilla flavour in beans is formed only during a careful curing process resulting in 2% vanillin (on dry weight basis) and over 170 other compounds with delicate sweet fragrance. The curing process has been conventionally developed over a few centuries in vanilla-growing countries, as an art rather than a science. Thus curing is the most crucial and laborious step in the entire process of natural vanilla production.

In the conventional curing process, the mature beans are blanched in hot water and subjected to a process involving periodic 'sunning' and 'sweating' where the former entails spreading the beans on blankets and exposing them to the sun for 2–3 h per day. During the remaining part of the day, the beans are wrapped in a blanket and allowed to 'sweat' indoors on wooden racks in a well-ventilated room, and the process is repeated for nearly a month followed by conditioning at room temperature for another 3–5 months. Thus there is an enormous handling activity indicating laborious steps and loss of flavour into the air. A large number of handlings for sunning and sweating generally result in a low-quality product. Due to these reasons, there are various attempts to modernize the curing process, which involves solar drying, oven drying and enzyme treatment (Dignum, Kerler, & Verpoorte, 2001). However, so far there has been no attempt to apply elicitors for curing vanilla beans.

Elicitors are compounds that trigger the increased production of pigments, flavones, phytoalexins and other defence related compounds. Elicitation has been found as an effective strategy for the induction and enhancement of secondary metabolites at a commercial scale. For example, synthesis of shikonin and its derivatives by suspension cultures of *Lithospermum erythrorhizon* was by the use of elicitors – agaro-pectins (Tabata & Fujita, 1985). In genetically transformed root cultures of red beet a significantly high productivity of 5-fold betalain was observed when pullulan was used and 4-fold higher pigment accumulated when cultures were treated with dry cell powder of *Penicillium notatum* (Savitha, Thimmaraju, Bhagyalakshmi, & Ravishankar, 2006). Peroxidase is one of the key enzymes in phenyl-propanoid (PP) pathway through which defence-related compounds are biosynthesized in higher plants. Significant elicitation in the activity of peroxidase was accomplished by the addition of the dry cell powder of *Candida versatalis* (3.5-fold higher than the control) or glutathione (3.44-fold) or *Rhizophus oligosporus* (3.09-fold) (Thimmaraju et al., 2006). Rao and Ravishankar (2000) listed biotransformations of a number of PP compounds to vanillin/vanillic acid. Nevertheless, failure of an elicitor to trigger a particular metabolic pathway does not necessarily establish its total in-efficacy. An elicitor may be ineffective under a combination of inappropriate conditions as well as unsuitable concentration of an elicitor. For example, the PP pathway was

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not induced in cultures of *V. planifolia* by yeast extract whereas the same elicitor induced phytoalexin in other cultures; the PP pathway in *V. planifolia* could however be triggered by using chitosan (Funk & Brodelius, 1990), indicating that a successful elicitation is a very challenging process requiring intense screening procedures.

While processing vanilla beans, the low yield of flavour compounds is also due to inefficient enzymatic conversion of the phenolic glycosides (Ruiz-Teran, Perez-Amador, & Lopez-Munguia, 2001). Although flavour substrates and relevant enzymes co-exist in vanilla beans, low yields of flavour have been attributed to the compartmentalization of substrate and the enzyme (Odoux, Escoute, Verdeil, & Brillouet, 2003). The total picture of chemical reactions occurring while curing vanilla beans is not fully understood. However, the enzymes playing important roles in the flavour formation have been identified. β-Glucosidase (β-GLUC) is known to hydrolyze the glucovanillin, the non-aromatic precursor, resulting in the release of the major flavouring component – vanillin (Odoux et al., 2003). Peroxidase (POD) activity in vanilla beans is found to be quite high even during curing (Sreedhar, Roohie, Venkatachalam, Narayan, & Bhagyalakshmi, 2007) and hence may be implicated in oxidation/reduction of flavour-forming compounds. For vanilla flavour development one may also envisage the involvement of cell wall degrading enzymes like cellulase, hemicellulase and pectinase by way of breaking down the cell walls and making the flavour substrates available for the enzyme to act (Ranadive, 1992). Accordingly, the treatment of vanilla beans with additional enzymes resulted in enhanced flavour formation. For example, successive treatment of green vanilla beans with pectinase and β-glucosidase followed by curing was found to result in 6% vanillin compared to 1.75% in traditionally cured beans (Mane & Zucca, 1999). In one study it was observed that a two step enzymatic reaction system with Viscozyme® (mixture of cellulase, arabinase, hemicellulase, xylanase and pectinase from *Aspergillus*) and Celluclast-(Novo)® (cellulase from *Trichoderma reseii*) resulted in an increase of glucovanillin extraction and its further conversion to vanillin to an extent of 3.13-folds (Ruiz-Teran et al., 2001).

With this background, the present study was undertaken to find out first whether the elicitors are useful for hastening the process of flavour formation, and if so, whether the levels of flavour turnover are corroborative with the levels of endogenous enzymes of the beans or with those of the elicitors, or both. The present study also addresses whether the flavour compounds thus generated are comparable with the profile and sensorial properties of conventionally cured beans.

2. Materials and methods

2.1. Chemicals

Authentic vanillin, vanillic acid, *p*-hydroxybenzoic acid, and *p*-hydroxybenzaldehyde, and other standard compounds were obtained from Sigma–Aldrich, USA. Horse-radish peroxidase was obtained from MP Biomedicals, USA. Solvents used were of HPLC-grade methanol (Ranbaxy, India), triple distilled water and AR grade glacial acetic acid (Qualigens, India). Ethanol was distilled before use.

2.2. Vanilla beans

Mature beans of vanilla ranging in length 4–5 in. (second grade) were obtained from the growers during the second phase of harvesting season (early December), transported within 24 h to the laboratory and used for the study. For the comparison of the quality of flavour formed after elicitor treatment, the conventionally cured beans from the market were used in which the curing had been done by intermittent sun drying and sweating process for 3–6 months (Dignum et al., 2001).

2.3. Selection, preparation and analyses of elicitors

2.3.1. Selection of elicitors

First the dry cell powders of several food-grade microbes such as *Aspergillus niger*, *C. versatalis*, *Rhizopus* species, *Lactobacillus heleviticus*, *Trichoderma* species, *Saccharomyces cerevisiae*; cell powders from seedlings of red beet, radish, carrot, turnip and French beans were screened for their efficacy in enhancing the flavour formation in a 10 day period. This resulted in the selection of three elicitors – *A. niger* (CFR-1038), *S. cerevisiae* (CFR-101) and red beet seedling powder for the present study. In addition to their elicitation effects, these elicitors may also serve as the sources of enzymes. Since three major enzymes were known for their involvement in converting the substrates to vanilla flavour, the elicitors were screened for the levels of β-glucosidase (β-GLUC), cellulase (CEL) and peroxidase (POD) enzymes, and their respective levels are presented in Table 1. The required quantities of *A. niger* and *S. cerevisiae* were prepared based on prior reports (Funk & Brodelius, 1990; Rao & Ravishankar, 2000; Thimmaraju, Bhagyalakshmi, & Ravishankar, 2004) and the preparation of red beet elicitor is briefly presented in the following.

Red beet elicitor. Seeds of red beet variety "Ruby Queen" of Indo-American Hybrid Seeds (India) were purchased from local horticultural shop and sown in perforated plastic trays filled with soil mixture (red soil: sand: garden manure in equal parts) and moderately watered with tap water. Within two weeks, the seeds germinated producing 2–3 cm long roots. The seedlings were carefully removed from the soil, the aerial portions of seedlings were cut off and the roots were separated from the soil, washed in running tap water followed with several rinses in distilled water.

2.3.2. Preparation of dry cell powder (DCP)

The well-grown microbial cultures and seedling roots were washed several times with sterile distilled water. Surface-bound water was fairly removed by applying vacuum pressure to the filtered residue, the biomass was weighed and divided into two parts; while one part was dried by washing quickly with chilled acetone, the other part was dried by lyophilization. All the elicitors were then powdered using pre-chilled pestle and mortar and further stored at 0 °C until use. Such elicitor preparations have been found highly useful in eliciting secondary metabolites in various systems (Savitha et al., 2006; Thimmaraju et al., 2004) as well as the associated enzyme (Thimmaraju et al., 2005).

2.3.3. Enzyme activities in dry cell powder

Peroxidase. Buffer of pH 6.0 was prepared using 0.2 M sodium phosphate, containing 1 mM Dithiothreitol and 0.1 mM Phenyl Methyl Sulfonyl Fluoride. The DCPs, 100 mg each were used to extract POD by soaking and crushing in 1 ml of buffer. The homogenate thus obtained was centrifuged at 12,000 rpm twice and the supernatant was used for further assay of peroxidase activity. The POD assay was done following the method described by Wititsuwannakul, Wititsuwannakul, Sattaysevana, & Pasitkul, 1997. The results were expressed as units per gram dry weight. Activity was quantified on the basis of standard curve of horseradish POD enzyme (MP Biomedicals, USA). One unit of enzyme activity refers to the rate of change of 1 OD per minute. The specific activity was expressed as enzyme units per milligram protein. POD activity in DCPs was mainly uniform during a thermal stability test at 38 °C for 40 days.

Cellulase. CSE activity was determined by measuring the reducing groups released from carboxymethyl cellulose (CMC, Sigma,

Table 1
Activities (U g^{-1} dry weight) of enzymes in elicitor powders processed either by lyophilization (L) or acetone-wash (A) and in fresh vanilla beans

Enzyme → Elicitor name	Cellulase		β-Glucosidase		Peroxidase	
	L	A	L	A	L	A
Aspergillus niger	3.5 ± 0.13	15.7 ± 0.42	18.7 ± 0.45	5.8 ± 0.67	1085 ± 41	1206 ± 25
Saccharomyces cerevisiae (yeast)	19.0 ± 1.64	14.0 ± 0.68	Tr	Tr	1507 ± 37	1350 ± 44
Red beet seedling root	3.5 ± 0.1	3.2 ± 0.12	0	0	92,800 ± 280	98,500 ± 260
Fresh vanilla beans[a]	0.74 ± 0.12	172.3 ± 9.3	1125 ± 29			

Tr: Traces.
[a] Data presented at 25% moisture level.

USA) as indicated in our earlier report (Sreedhar et al., 2007). 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 5.0) incubated at 37 °C for 1 h. One unit (U) is defined as the amount of enzyme that catalyzed the formation of 1 μM reducing group min^{-1}. The activity of this enzyme at 38 °C was found to increase by 10% on 10th day reaching the initial level by 20th day and diminishing by 5% on 30th day remaining unchanged by 40th day.

β-*Glucosidase.* Although a recent report indicated the use of sodium phosphate buffer of pH 7.0 at 40 °C (Odoux et al., 2003), our standardization indicated that citrate buffer was ideal for the assay of this enzyme. Therefore, the activity of β-GLUC was determined at 30 °C in citrate phosphate buffered medium of pH 5.0 using *p*-nitrophenol glucopyranoside as the substrate. Here, the *p*-nitrophenol released, after the glucose is hydrolyzed by the enzyme, was 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 (Spagna, Barbagallo, Palmeri, Restuccia, & Giudici, 2002). The hydrolysis of 1 μmol of substrate min^{-1} was recorded as 1 unit of activity and quantified on the basis of $\varepsilon = 18{,}500\ mol^{-1}\ cm^{-1}$ reading at 400 nm and the data was recorded in each case for 3 min of 30 s intervals. The activity of this enzyme at 38 °C was found to decrease by 10% on 10th day diminishing further by 5% on after every 10 days.

2.4. Treatments

The vanilla beans were blanched at 63 °C for 3 min as explained earlier (Sreedhar et al., 2007). To bring the elicitors in contact with the living cells of the beans, the beans were scarified lengthwise with a brush having fine stainless steel bristles (automation for blanching and scarification is also possible). Different sets, each having 20 scarified beans (×6), were kept on two-layered butter paper sheet and sprinkled with different elicitor powders at the rate of 5 mg DCP per bean. The treated beans (20 in each bundle) were wrapped and incubated in an oven at 38 ± 1 °C as reported (Sreedhar et al., 2007).

2.5. Enzyme assays in vanilla beans

2.5.1. Enzyme quantification

Earlier studies indicated the involvement of three major enzymes during the curing of vanilla beans: the involvement of β-GLUC for catalyzing the conversion of glucovanillin and other glycosides to vanillin and respective flavour compounds, cellulase (CSE) for cell-wall degradation assisting the permeabilization of β-GLUC from the surface of the beans to the center, and POD in various bioconversions of phenyl-propanoid compounds (Dignum et al., 2001). Therefore, the activities of these three enzymes were followed in the fresh beans (at the time of initiation of the experiment) and at specific intervals throughout the curing period (after different treatments) by following the methods as explained in the previous sections. For this, three beans from each of the triplicate bundles were randomly picked, weighed and extracted by chopping of beans into 1 cm pieces followed by crushing at 4 °C with 50 ml of respective buffers and centrifuged twice at 5000*g* for 15 min, and the supernatant was used as the enzyme source.

2.5.2. Tissue printing

Activities in the fresh beans were also checked by tissue printing method where cross sections of beans were obtained from distal end (away from the petiole), middle region and proximal end (near the petiole) and immediately and carefully placed on nitrocellulose paper supported on sterile blotter sheets. After the sap from the sections imbibed into nitrocellulose, the latter was processed at 4 °C in buffer solutions containing substrates for the enzyme in question. For peroxidase, 100 ml of (at 4 °C) sodium phosphate buffer (pH 6.0) containing 10 ml of 0.25% *o*-dianisidine dihydrochloride and 10 ml of 1% H_2O_2 was used. For β-glucosidase, 100 ml of reaction solution containing per ml concentration of 100 μl of 0.1 M sodium citrate buffer (pH 5) and 100 μl of 0.0055 M *p*-nitrophenol β-D-glucopyranoside (Spagna et al., 2002) was used. For control, the prints of bean slices were processed in respective buffers without substrate. After 10 min, each nitrocellulose sheet was kept on multilayered sterile blotters and the colour developed was photographed. Localization of enzymes in bean tissues was re-checked by keeping the thin sections on a glass slide and directly adding the respective buffer solutions with or without substrate. The enzymatic reactions were immediately photographed.

2.6. Protein estimation

Total protein was extracted from the beans and estimated by macro-Kjeldahl method (total nitrogen × 6.25) as the phenolic compounds hindered the estimation by Lowry's method. The data was normalized and taken as % weight of beans with 25% moisture content.

2.7. Analyses of flavour

2.7.1. Preparation of reference standards

Preparations of standards and conditions for HPLC analysis were done as per an earlier report (Sreedhar et al., 2007).

2.7.2. Extraction of flavour compounds

The flavouring components from vanilla beans were extracted as described earlier (Sreedhar et al., 2007) where, triplicate samples (20 beans each) of cured vanilla beans were finely chopped and 10 g of chopped beans were first finely crushed in liquid nitrogen; the powder thus obtained was extracted with 75 ml of 44% aqueous ethanol at 45 °C in stoppered conical flasks. The mixture was occasionally agitated and after 48 h, the mixture was 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 m) to remove coarse particles, and a clear aliquot was used for HPLC analyses (Sreedhar et al., 2007).

2.8. Sensorial properties

Sensory evaluations were carried out in seven separate booths maintained at a temperature of 22 ± 2°C with 45 ± 5% of relative humidity with fluorescent lights, which was equivalent to day light illumination. The Quantitative Descriptive Analysis (QDA) method (Stone & Sidel, 1998) used for profiling sensory attributes consisted of 15 cm line scale wherein 1.25 cm was anchored as low and 13.75 cm as high.

2.8.1. Odour profile analysis of vanilla pods

One gram of vanilla pod was taken in 250 ml conical flask with stopper. Panelists were trained to sniff the headspace and mark the intensity of odour notes in the scorecard. The scorecards were decoded and mean values of the attributes were calculated from three separate analyses. The profiles were generated as spider web diagram by plotting attributes versus mean scores.

2.8.2. Panel training

A group of 12–15 panelists were trained over three sessions for descriptive sensory analysis. The members of the staff were familiar with sensory analysis techniques used in plantation products and flavour technology and related fields. The training included development of a common lexicon of the sensory attributes in evaluation. For this, a vanilla flavour lexicon wheel procedure was used (Hariom, Shyamala, Prakash, & Bhat, 2006). The common descriptors selected by at least one-third of the panel and few important descriptors cited in the literature were utilized in the development of the scorecard. In order to assist panelists in the selection of descriptors, dominant flavour notes of vanilla and appropriately diluted reference compounds corresponding to the flavour notes (Hariom et al., 2006) were provided. The panelist evaluated the vanilla extracts in a group and recorded the perceived attributes individually. Following this, an open discussion was held to reach to an agreement on appropriate descriptors and the threshold levels were decided for plotting the graph.

2.9. Statistical analyses

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 $p \leqslant 0.05$.

3. Results

3.1. Total protein

There was no significant change in the level of protein during the treatment period in vanilla beans throughout the curing period. In all treatments, the initial protein content of 5.7% was found and the cured beans showed 5.55% protein indicating an insignificant loss during the treatment period of 40 days (data not shown separately).

3.2. The enzymes

The levels of activities of the three enzymes followed in the present study are summarized in Table 1. POD was dominant in all the elicitor powders, with a high level of activity (98,500 ± 260 U g^{-1} DW) in acetone-dried powder of red beet seedling root (BSR) than in the lyophilized counterpart (92,800 ± 280 U g^{-1} DW). The other elicitors, *A. niger* and *S. cerevisiae*, showed considerably high activity of POD (over 1000 U g^{-1} DW), however, the values were significantly lower than those in BSR elicitors. The activity of β-GLUC was not traceable in BSR whereas some activities of CSE and POD were found in all the elicitors. Even in case of vanilla beans, the fresh ones showed high POD activity (1125 ± 29 U g^{-1} tissue) distributed throughout the fruit, although the core and the distal region showed higher activity of POD than the surface and the proximal (petiolar) region. Assay of nitrocellulose membranes on which tissue prints were obtained also revealed similar results with good intensity of colour developed when substrate for POD was used. In contrast, the activity of β-GLUC was feeble in tissue slices indicated by slight increase in yellow colour with no results in tissue prints. When assayed, the activity of β-GLUC was 172.3 ± 9.3 U g^{-1} tissue (at 25% moisture level) (Table 1).

Throughout the curing period, with and without elicitor treatment, the enzyme activities in beans showed large differences (Table 2). The level of β-GLUC, which was 172.3 ± 9.3 U g^{-1} tissue at initial level, increased to 213.7 U g^{-1} tissue on the 10th day with a further increase to 358.1 U g^{-1} tissue on the 20th day. However, there was a steady decline towards the end of the curing period. POD activity significantly decreased to 995.4 U g^{-1} tissue from 1125 ± 29 U g^{-1} tissue at initial level during curing with a narrow increase on the 20th day and a significant drop on the 30th day following a slight increase on 40th day (Table 2). The traces of cellulase activity present in the beans on 10th day of curing steadily declined towards the end.

Upon elicitor treatment, there was a steady increase in the activity of β-GLUC, especially in the acetone dried *A. niger*-treated beans, with a highest activity of 513 U g^{-1} tissue on the 40th day of curing. A higher activity was recorded in acetone-dried elicitor treated material than that treated with lyophilized material (Table 2). Acetone-dried BSR (BSR-A) was also an efficient enhancer of β-

Table 2
Levels of activities of different enzymes in control and elicitor-treated vanilla beans during various stages of curing[a]

Elicitor	10 DAC	20 DAC	30 DAC	40 DAC
Peroxidase (U g^{-1} tissue[b])				
Control[c]	995 b	1038 cd	1004 b	1011 bc
A. niger-A	1045 b	1288 b	1097 b	1157 ab
A. niger-L	1010 b	1235 b	1239 ab	1044 b
BSR-A	1323 a	1504 a	1327 a	567 d
BSR-L	1235 ab	1408 ab	814 c	707 cd
Yeast-A	969 b	1052 cd	1081 b	1160 ab
Yeast-L	1001 b	1030 cd	1073 b	1363 a
β-Glucosidase (U g^{-1} tissue[b])				
Control[c]	214 c	358 ab	319 bc	247 cd
A. niger-A	237 b	396 a	464 a	513 a
A. niger-L	219 bc	289 bc	382 b	317 bc
BSR-A	249 a	198 d	222 cd	234 cd
BSR-L	195 b	241 cd	210 cd	207 cd
Yeast-A	228 b	264 c	240 cd	180 d
Yeast-L	224 b c	259 c	239 cd	209 cd
Cellulase (U g^{-1} tissue[b])				
Control[c]	2.69 d	2.09 d	1.57d	0.98 cd
A. niger-A	2.97 cd	3.05 bc	2.54 c	1.07 c
A. niger-L	4.10 a	4.68 a	4.89 a	2.24 a
BSR-A	2.19 de	1.90 d	1.74 d	0.71 d
BSR-L	1.72 e	2.28 cd	2.00 cd	0.68 d
Yeast-A	2.60 d	2.82 c	2.43 c	0.97 cd
Yeast-L	2.43 d	2.94 c	1.82 cd	0.65 d

DAC, days after curing; A, acetone-dried; L, lyophilized; BSR, beet seedling root.
Data followed by different letters within each column are significantly different according to Duncan's multiple-range test at $p \leqslant 0.05$.
[a] Data are the mean value of three replicates.
[b] At 25% moisture level.
[c] Experiment-specific control where the beans were blanched and scarified.

GLUC activity in the beans as compared to the control. Yeast elicitors showed a narrow enhancement in β-GLUC only at initial stage with lower activities during further curing periods. However, significant increase as well as fluctuations in the activities of POD were observed when the beans were treated with *A. niger* and BSR-A, where the latter showed highest value on 20th day of curing with a steady decline later. The activity of CSE was very low (0.74 ± 0.12 U g^{-1} tissue) in the fresh vanilla beans (Table 1). During curing a three to four fold increase in activity was observed on 10th day and *A. niger* (lyophilized) showed a slightly higher activity on 20th and 30th day of curing (Table 2).

3.3. Formation of flavour compounds

The control beans on zero day contained 0.38% vanillin, which may be formed mainly during extraction because vanilla beans do not impart any flavour before curing. Other compounds were not traceable by HPLC on day zero. Fig. 1 shows the HPLC patterns

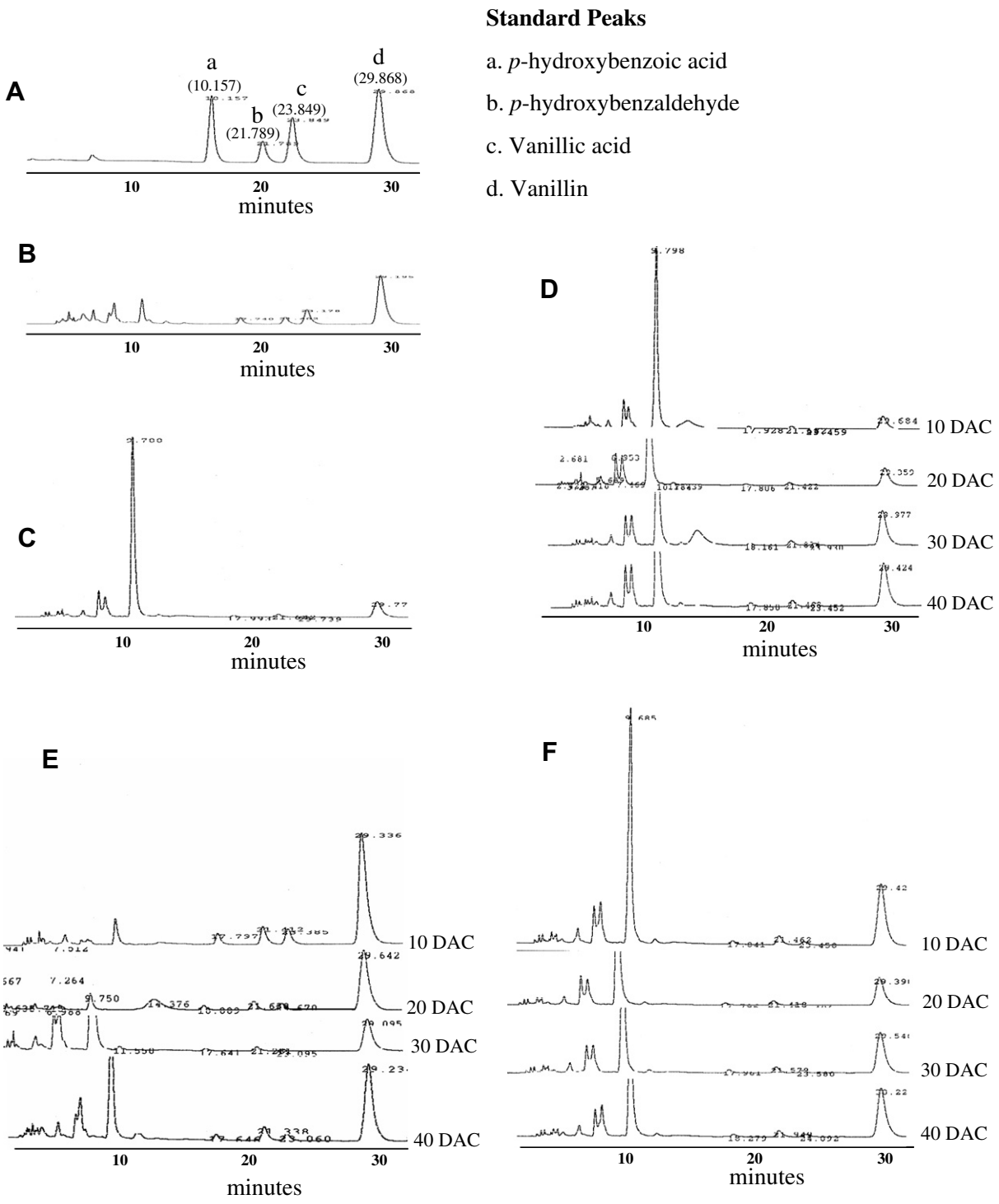

Fig. 1. HPLC patterns showing profiles of vanilla flavour compounds formed after 10 days of curing at 38 °C in elicitor-treated vanilla beans as compared with standards and control beans. (A) Standard compounds, (B) commercial sample, (C) green bean (uncured), (D) control (blanched and scarified), (E) treated with red beet seedling root powder-acetone dried, (F) treated with *A. niger* powder-acetone dried, DAC: days after curing.

Table 3
Concentrations of different flavour compounds in control and elicitor treated vanilla beans during different stages of curing[a]

Treatment	Vanillin	Vanillic acid	*p*-Hydroxy-benzaldehyde	*p*-Hydroxy-benzoic acid
		(Gram percent of bean weight[b])		
10 DAC				
Control[c]	1.58 d	0.003 c	0.003 c	0.001 cd
A. niger-A	2.02 c	0.006 ab	0.005 bc	0.004 b
A. niger-L	1.98 c	0.006 ab	0.003 c	0.006 a
BSR-A	2.65 a	0.007 a	0.006 b	0.003 bc
BSR-L	1.98 c	0.005 b	0.005 bc	0.002 c
Yeast-A	1.67 d	0.004 b	0.009 b	0.001 cd
Yeast-L	1.80 cd	0.003 c	0.010 a	0.002 c
20 DAC				
Control[c]	1.42 d	0.001 d	0.003 bc	0.001 cd
A. niger-A	1.65 bc	0.004 c	0.004 b	0.005 ab
A. niger-L	1.60 c	0.005 bc	0.000 d	0.004 b
BSR-A	1.95 a	0.005 bc	0.004 b	0.003 bc
BSR-L	1.89 ab	0.008 a	0.006 a	0.004 b
Yeast-A	1.58 c	0.006 b	0.002 c	0.00 cd
Yeast-L	1.58 c	0.005 bc	0.005 ab	0.006 a
30 DAC				
Control[c]	1.59 c	0.004 bc	0.003 c	0.001 c
A. niger-A	1.80 ab	0.004 bc	0.004 c	0.000 c
A. niger-L	1.45 d	0.006 ab	0.002 d	0.007 a
BSR-A	1.87 a	0.006 ab	0.007 b	0.005 bc
BSR-L	1.70 bc	0.008 a	0.010 a	0.006 ab
Yeast-A	1.62 c	0.00 c	0.005 bc	0.006 ab
Yeast-L	1.85 ab	0.007 ab	0.008 ab	0.008 a
40 DAC				
Control[c]	2.00 ab	0.009 b	0.005 cd	0.002 d
A. niger-A	1.85 bc	0.004 c	0.004 e	0.003 cd
A. niger-L	1.65 d	0.00 d	0.005 cd	0.005 bc
BSR-A	2.05 a	0.01 a	0.006 c	0.008 a
BSR-L	1.85 bc	0.008 b	0.008 a	0.004 c
Yeast-A	1.82 c	0.006 bc	0.007 b	0.005 bc
Yeast-L	1.75 cd	0.005 bc	0.005 d	0.006 b

DAC, days after curing; A, acetone-dried; L, lyophilized; BSR, beet seedling root.
Data followed by different letters within each column are significantly different according to Duncan's multiple-range test at $p \leqslant 0.05$.
[a] Data are the mean value of three replicates.
[b] At 25% moisture level.
[c] Experiment-specific control where the beans were blanched and scarified.

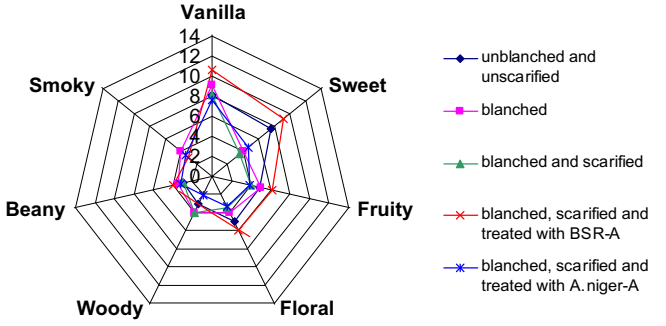

Fig. 2. Sensory profile of vanilla beans cured for 10 days with or without elicitors and different pre-treatments. The data represents average of atleast 12 panelists in three separate analyses.

of standard compounds, commercial sample, green bean (uncured), as well as those obtained from elicitor-treated bean samples cured for 10 days. The major compound, vanillin, was formed to an extent of nearly 1.58% in control beans on the 10th day. However, in the samples treated with elicitor of red beet seedling root prepared by acetone-drying method, a very high level of 2.65% of vanillin was observed on the 10th day itself (Table 3). This level of vanillin formation is 1.7-fold higher than control beans of this study on the 10th day and much higher (3.23-fold) than the conventionally cured sample (0.82%) (Sreedhar et al., 2007) and nearly 7-fold compared to zero day (0.38% in green bean). Nearly similar effects were observed in case of treatments with *A. niger* and BSR-L elicitors, all producing nearly 2% vanillin with 2-fold increase in vanillic acid and slight fluctuations in the levels of *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid. In the control sample itself there were significant fluctuations in the quantities of flavour compounds formed during the entire curing process, with a decline in vanillin after 20 days with a steady increase up to 40 days.

3.4. Sensory attributes

The observations made on odour profile of vanilla bean samples are shown in Fig. 2. In case of unblanched and unscarified treatment, vanilla note differed significantly ($p \leqslant 0.05$) from blanched beans and BSR-A treated beans (Fig. 2). Treatment with red beet seedling elicitor appeared to impart good sensory profile to the beans with high notes of vanilla, sweet and floral odour and low intensity of woody, beany and smoky notes.

4. Discussion

Elicitors are known to interfere with plant phenyl-propanoid (PP) compounds catalyzing lignification of plant cells. Since the natural vanilla flavour from cured beans of *V. planifolia* comprises over a hundred flavour molecules derived from the PP pathway, the effects of elicitors were studied, assuming that they might hasten the curing process by catalyzing relevant enzyme activities.

The data presented in Table 1 show significant differences in the activities of enzymes, indicating that the method used for elicitor preparation possibly influenced their characteristics. Peroxidase showed lesser sensitivity to processing conditions than other enzymes. Enzyme activity change due to processing conditions is widely known.

The major enzyme present in both vanilla beans and elicitors was the POD. The formation of very high levels of vanillin was also noted in the treatment with high levels of POD, which is indicative of the major role played by this enzyme in catalyzing the flavour pathway. Earlier studies have established that peroxidases (of Class III type) are widely distributed in higher plant cells. Plant PODs and their various isoenzymes have been proven responsible for a plethora of physiological functions where they preferentially use phenolics as electron donors resulting in the formation of oxidized phenolic compounds of brown colour (Hanum, 1997). Since their isoenzymes are diversely regulated (Welinder & Gajhede, 1993), one can expect wide variations as well as an array of the end product. Whereas the fungal peroxidases (Class II type) are known to act at extra-cellular level and are mainly involved in lignin degradation; often contributing for the re-formation of vanillin (Hanum, 1997; Priefert et al., 2001). Ferulic acid (an iso-lignin) occurs abundantly in most of the plant cells including vanilla beans. Earlier labeling studies have established its preferential incorporation into vanillin over other closely related substrates leading to the higher turnover of vanillin (Zenk, 1965). Thus, there is a good relationship between the levels of POD (*in situ* + that of elicitor) and the levels of vanilla flavour compounds where the enzyme could probably catalyze the inter-conversions of the flavour precursors resulting in the formation of appropriate substrates for the action of the other key enzyme-β-GLUC.

The potential of *A. niger* to convert natural precursors of vanilla flavour, *i.e.,* isoeugenol to vanillin (Abraham, Arfmann, Stumpf, Washausen, & Kieslich, 1988), ferulic acid into vanillic acid (Bonnin, Lesage-Meessen, Asther, & Thibault, 1999) and vanillic acid to vanillin (Priefert et al., 2001), is well known. *A. niger* has also been described as a ferulic acid-degrading organism (Labuda, Goers, & Keon, 1992). Commercial exploitation of *A. niger* for the conversion of ferulic acid to vanillic acid has been described (Lesage-Meessen et al., 1999). The treatment with the dry cell powder of *A. niger,* particularly the acetone-dried powder, significantly enhanced vanillin on 10th day itself, which was nearly 1.3-fold higher than the blanched and scarified control, and 2.46-fold higher than the traditional method of blanching (Sreedhar et al., 2007). Noteworthy improvements in the other flavour components were also observed during this period. Lyophilized *A. niger* powder was more effective in enhancing vanillic acid and benzoic acid steadily up to 30th day.

A few studies have demonstrated the direct involvement of β-GLUC as the key enzyme for the de-glucosylation of flavour substrates, which is also controversial due to compartmentalization of the enzyme from the substrate (Dignum et al., 2001; Odoux, 2006; Odoux et al., 2003; Havken-Frenkel et al., 2004). β-GLUC is also expected to enhance flavour extractability by hydrolysis of cell wall components (Ruiz-Teran et al., 2001). Nevertheless the present study and our previous report (Sreedhar et al., 2007) indicate that the high levels of POD of the beans and that in the elicitor may also be involved in the quantum turn over of the flavour molecules, although a deeper study is needed to unequivocally establish the same.

It is interesting to note that there is a decline in the level of vanillin on 20th and 30th days. These changes may again be attributed to the catalytic activities of POD followed by β-GLUC, where the latter systematically catalyzes the de-glucosylation whereas the former may be implicated in a cascade of redox reactions. Plant PODs (POD class III), (both from vanilla beans and red beet seedling root-derived) having high redox potential may not only build up but also degrade vanilla flavour compounds. Therefore, in a mixture of phenolics, the radicals of phenolics that are good substrates for POD can oxidize a poor electron donor molecule for peroxidase, rapidly. Thus, even the un-preferred substrate is indirectly catalyzed by POD, bringing about fluctuations in the flavour profile as observed in the present study. However, the fungal PODs are known to work the other way, where they depolymerize lignin to form vanillin (Hammel et al., 1993; Kirk & Farrell, 1987; Priefert et al., 2001; Ten Have, Rietjens, Hartmans, Swarts, & Field, 1998). Thus the highest turnover of vanillin in the red beet seedling powder or *A. niger*-treatments (being rich in POD) may be attributed to the re-conversion of degraded vanillin and similar *iso*-lignin molecules.

Beans treated with red beet seedling powder showed a better sensory profile with high notes of vanilla, sweet, and floral and low woody, beany and smoky notes which is a characteristic feature of good quality vanilla (Hariom et al., 2006). Attributes such as fruity, floral, woody and beany notes did not differ significantly from the others, which might be due to the use of the higher concentrations of the sample which might have masked the subtle differences.

5. Conclusion

The present study has clearly demonstrated that for developing a process for the production of vanilla flavour compounds from vanilla beans, one need to carefully control the process parameters with periodical monitoring for flavour compounds and terminating the reaction as desired because the endogenous enzyme may divert the flavour molecules towards lignin biogenesis. This study has also shown that the HPLC profile as well as the sensorial properties of the beans cured just for 10 days with elicitor were characteristically similar to the conventionally cured beans indicating that the present finding holds great promise for further application in vanilla bean curing.

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