NMR-based metabolomic characterization of Vanilla planifolia

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Chapter 7

Metabolomic analysis of *V. planifolia* organogenic calli during shoot differentiation

Abstract

Vanilla *plants are conventionally propagated by stem cuttings. Regeneration and in vitro mass multiplication are proposed as an alternative to minimize damage to mother plants. The molecular markers responsible for shoot induction have been studied at the stage where no differentiating parts were visible on organogenic calli. Metabolic analysis of calli samples using NMR spectroscopy showed the importance of numerous compounds related to sugar mobilization and nitrogen metabolism. Accumulation of phenolic compounds was also enhanced during shoot differentiation. These results might contribute to elucidate the complex mechanism of vanilla callus differentiation and subsequent shoot formation. Moreover, glucoside A and glucoside B were identified for the first time in vanilla tissue culture. Their degradation in specialized tissue (i.e. young green beans) could contribute to the biosynthesis of vanillin glucoside.*

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7.1 Introduction

The genus *Vanilla* belongs to the family Orchidaceae, which consists of more than 110 described species (Bory et al., 2008c). *Vanilla planifolia* Andrews, *V. tahitensis* and V. *pompona* are the commercially important species cultivated for the production of natural vanilla flavor (Havkin-Frenkel and Dorn, 1997). *Vanilla planifolia* is preferred to the two other species for its flavor (Ehlers and Pfister, 1997; Sreedhar et al., 2007).

The seeds of vanilla do not usually germinate and hence the plants are propagated by vegetative means through stem cuttings resulting in a slow rate of multiplication. Moreover, this method is not economical since the collection of stem cuttings leads to the interruption of growth of the mother plant, resulting is a yield reduction which can be minimized by in vitro regeneration and mass multiplication. Hence, the in vitro multiplication of *V. planifolia* using shoot tip and axillary buds has been frequently reported (Geetha and Shetty, 2000; Kalimuthu et al., 2006). Thus, cultivated plants are genotypically identical but all could be sensitive to the aggressiveness of virulent soil pathogens (Havkin-Frenkel and Belanger, 2007). Somaclonal variation can be induced if regeneration includes callus culture (Jain, 2001). Unfortunately, only a few reports are available on plantlet regeneration through multiple adventitious shoot differentiation from *V. planifolia* callus culture (Janarthanam and Seshadri, 2008). Mass production of *V. planifolia* through indirect shoot organogenesis is rare and there are numerous biological unknowns about the induction of this morphogenesis.

Organogenesis and somatic embryogenesis are both morphogenetic processes leading to plantlet regeneration. It is postulated that control over embryogenic development occurs at the protein and metabolite levels (Dowlatabadi et al., 2009). The ultimate goal of metabolomics is both qualitative and quantitative analysis of all metabolites in an organism or tissue (Verpoorte et al., 2007). It can provide a broad view of the biochemical status of an organism in interaction with its environment. Chemical analysis techniques applied to metabolomic profiling involves Nuclear Magnetic Resonance spectroscopy (NMR) since NMR can provide direct molecular structure information in the analysis of complex biological mixtures such as cell growth media (Dowlatabadi et al., 2009) or plant extracts (Hendrawati et al., 2006; Jahangir et al., 2008a).

Metabolomic analysis represents a great potential for exploring the biological processes involved in response to environmental changes such as the effect of plant hormones in

medium culture. In this study, we have used organogenic calli from *V. planifolia* protocorm to investigate their metabolic profile changes during earlier stages of shoot differentiation by ^{1}H-NMR spectroscopy. We have identified several metabolites whose expression differed during early events of shoot organogenesis. Liquid chromatography-mass spectrometry (LC-MS) has been used to confirm metabolite identification.

7.2 Materials and Methods

7.2.1 Plant material

Seven-months-old green pods of *V. planifolia* (flavour elite, Accession number 13B3) were collected and immediately disinfected in 50% hydrogen peroxide solution. This pre-sterilization treatment was followed by complete sterilization in the laminar airflow cabinet using a protocol established by Kodja et al. (1998). The seeds were then removed from the pod, detached and 200-300 seeds were spread out in 9 cm Petri-dishes. These seeds were then cultured in the dark on a germination medium composed with basal medium (BM) containing TDZ (**Table 10**) for the production of protocorm like structures (PLS). The basal medium was composed of macro- and microelements of Murashige and Skoog (1962), vitamins of Morel and Wetmore (1951) and 30g/L of sucrose. This basal medium (BM) was supplemented with different growth regulators for callus induction from PLS, callus maintenance, differentiation, multiplication and elongation of shoots from calli and rooting of shoots (**Table 10**). Maintenance of calli was carried out by subculture on A4 medium at 21-days intervals for a six-month period. The calli were transferred to A10 medium to induce differentiation, multiplication and elongation of shoots after this stabilization period. Calli on A10 medium, (CA10) were cultivated during 4 months without subculture. Morphological and cytological and/or biochemical studies were effected on day 15 (d15), day 20 (d20), day 30 (d30), day 60 (d60), day 75 (d75), day 90 (d90) and day 120 (d120). In parallel and as a control, calli were cultured on A4 medium (CA4) from day 15 until day 120 for the same morphological, cytological and/or biochemical analyses to check if the expected shoot formation had been induced onto A4 medium. Culture conditions of calli induction from PLS to rooting of shoots consisted of 16 h photoperiod maintained by white cooled fluorescent tubes (NL36w/830) providing a light intensity of 55µmol.m^{2}.s^{-1} in a growth room set at 25 ± 1°C. All culture media were autoclaved at 120°C after adjusting the pH to 5.8 by using 1M KOH.

Table 10: Procedure of plantlet regeneration from callus derived protocorm of *V. planifolia*. In the same way as the experiment of shoot differentiation and multiplication on A10 medium with CA10 calli, organogenic calli were also cultured on A4 medium (CA4 calli) as a control. A4 medium and callus maintenance medium were identical.

Plant growth regulators in basal medium (BM)	Stage of plantet micropropagation				
	PLS germination	Callus induction medium	Organogenic callus culture on callus maintenance medium	Shoot differentiation, multiplication and elongation on A10 medium	Rooting on A5 medium
Thidiazuron (TDZ)	0.5 mg/L	0.5 mg/L	0.3 mg/L	0	0
Indole-3-acetic acid (IAA)	0	0.5 mg/L	0.5 mg/L	0	0
α–Naphtalene acetic acid (NAA)	0	0	0	0.5 mg/L	0
Phytagel	7.5g/L	7.5g/L	7.5g/L	7.5g/L	7.5g/L
Other features					
Container	Petri disk	Petri disk	tube	Flask	tube
Medium volume	20 mL	20 mL	20 mL	100 mL	20 mL
Subcultures	No	No	Every 21 days	No	No
Culture time	4 - 6 months	2 months	6 months	4 months	2 months

7.2.2 Histology and biochemistry

To confirm the occurrence of indirect shoot organogenesis, CA10 samples were collected 15, 20 and 30 days after transfer of calli onto A10 medium and CA10 d15, CA10 d20 and CA10 d30 calli were studied. Calli were fixed in formol 4% (Sigma-Aldrich), for 24 h at 4°C, transferred to automat system (Leica ASP 300) and then dehydrated under vacuum through a graded ethanol series (90%, 95, 100, 100, 100, 100%) for 1h each time; three times in methylcyclohexane (Sigma-Aldrich) for 1 h each time and embedded in paraffin (melting point: 58°C) after two immersions of 1 h for each. Control samples were removed after 15, 20 and 30 days from A4 medium and samples CA4 d15, CA4 d20 and CA4 d30 were respectively fixed as described above. Serials sections of 4µm thickness were

obtained with rotary ultramicrotome (Leica RM 2125 RT, Germany). For starch, other carbohydrates and nuclei detection, callus sections were stained in 0.5% (v/v) periodic acid (Sigma-Aldrich) for 10 min and 20 min in Schiff' reagent. Callus sections were rinsed with distilled H_2O and dehydrated in 100% ethanol followed by quick immersion in toluene (Sigma–Aldrich). Sections were stretched and mounted on glass slides (SuperFrost®, Menzel-Glaser) and were examined under Nikon's Eclipse TE 2000-U microscope. Photographs were taken using a digital camera of the same microscope.

7.2.3 NMR Analysis

Extraction

In order to study the metabolic changes during the earlier stages of shoot differentiation, CA4 d15 and CA10 d15 were used for the metabolomic analysis by ^{1}H NMR spectroscopy. The whole-size callus of four CA4 d15 calli and of four CA10 d15 calli was employed. For each callus, freeze-dried material (50 mg) was transferred to a 2 mL microtube. A volume of 1.5 mL of a mixture of KH_2PO_4 buffer (pH 6.0) in D_2O containing 0.05% trimethylsilylpropionic acid sodium salt (TMSP, w/w) and CH_3OH-d_4 (1:1) was added to the callus samples. The mixture was vortexed at room temperature for 1 min, ultrasonicated for 20 min, and centrifuged at 13000 rpm for 10 min. An aliquot of 0.8 mL was used for NMR analysis.

Measurements

^{1}H-NMR, 2D-*J* resolved, ^{1}H-^{1}H correlated spectroscopy (COSY), and heteronuclear multiple bonds coherence (HMBC) spectra were recorded at 25 ºC on a 600 MHz Bruker AV 600 spectrometer equipped with cryo-probe operating at a proton NMR frequency of 600.13 MHz. CH_3OH-d_4 was used as the internal lock. Each 1H-NMR spectrum consisted of 128 scans requiring 10 min acquisition time with the following parameters: 0.25 Hz/point, pulse width (PW) = 30° (10.8 µsec), and relaxation delay (RD) = 1.5 sec. A presaturation sequence was used to suppress the residual H_2O signal with low power selective irradiation at the H_2O frequency during the recycle delay. FIDs were Fourier transformed with LB = 0.3 Hz and the spectra were zero filled to 32 K points. The resulting spectra were manually phased and baseline corrected, and calibrated to TMSP at 0.0 ppm,

using Topspin (v. 2.1, Bruker Biospin). All the 2D NMR parameters were the same as in our previous reports (Jahangir et al., 2008a).

Data analysis

^{1}H-NMR spectra were automatically reduced to ASCII files using AMIX (v. 3.7, Bruker Biospin). Spectral intensities were scaled to total intensity TMSP and reduced to integrated regions of equal width (0.04 ppm) corresponding to the region of δ 0.30 – δ 10.02. The region of δ 4.70 - δ 5.00 was excluded from the analysis because of the residual signal of H_2O as well as δ 3.28 - δ 3.40 for residual CH_3OH-d_4. Principal component analysis (PCA) were performed with the SIMCA-P software (v. 11.0, Umetrics, Umeå, Sweden) using Unit Variance (UV) scaling method. The *t*-test for ^{1}H-NMR signals were performed using Multi-Experiment Viewer (v. 4.0) (Saeed et al., 2003).

7.2.4 LC-MS procedure

Extraction

In order to confirm the ^{1}H-NMR results, the same calli samples were analyzed by LC-MS. Freeze-dried samples (50 mg) were transferred into a 2 mL microtube. A volume of 1.5 mL of CH_3OH and H_2O (1:1) was added to the callus samples. The mixture was vortexed at room temperature for 1 min, ultrasonicated for 20 min and centrifuged at 13000 rpm for 10 min. The supernatant was then analysed with LC-MS.

LC-MS Analysis

The LC system employed was an Agilent CPL/SM 1100 series (Massy, France) equipped with LC/MSD Chemstation software, degasser G1322A, binary pump G1312A, autosampler G1313A, thermostated column oven G1316A, diode array detection system G1315B to monitor at all wavelengths from 200 to 400 nm, and MSD/VL mass spectrometer with APCI source. For the column, a LiChrospher 100 RP-18 (250 x 4.6 mm i.d., s-5, 5 µm) (Merck, Darmstadt, Germany), joined with a guard column LichroCART 4-4 (Merck), was used at 35°C. Gradient elution was performed with solution A, composed of 90% H_2O at 0.1% CH_3COOH (pH 3.3) and 10% CH_3OH, and solution B, comprising 70% CH_3OH, delivered at a flow rate of 1.0 mL/min as follows: initially 100% of solution A; for

the next 15 min, 70% A; for another 30 min, 65% A; for another 20 min, 60% A; for another 5 min, 5% A; and finally 0% A for 25 min. The APCI mass spectrometer conditions were as follows: negative (or positive mode if necessary) ion mode; fragmentation voltage, 70 V; capillary voltage, 4000 V; vaporizer temperature, 350°C; corona current, 15 µA; drying gas (nitrogen) flow, 11 mL min-1; nebulizer pressure, 60 psig; drying gas temperature, 350°C; mode scan, 10-1000 uma. The injection volume for the extract was 10 µl. For the polyphenol analysis, a library including 100 phenolic acids, catechins, flavonoids, and simple polyphenols was first made. The library was composed of HPLC retention times and UV-DAD spectra of aglycons, and a calibration table was constructed for each compound. The mass spectrum of each reference compound was also recorded and used to confirm identification. The internal standard used was *p*-formylbenzoic acid methyl ester (360 µmol). The callus extracts were analyzed using the same HPLC system. The polyphenols were identified on the basis of their retention times, UV-DAD spectra, and APCI mass spectra and quantified according to the calibration table.

7.3 Results

7.3.1 Adventitious shoots and in vitro plant formation

In vitro germination was observed after three months in culture with highest percentages between the fourth and sixth months. However, a weak germination percentage was observed (8%), with the formation of a massive structure originating from each seed embryo. This structure lengthened and subsequently developed into a protocorm (Protocorm Like Structure, PLS) showing an apical meristem and root primordium covered with rhizoids (**Figure 52a**). These PLS have developed calli after 2-4 weeks of culture on the callus induction medium (**Figure 52b**). Subculture to fresh medium was done at 21-day intervals for a period of six months (**Table 10**). After this period, the calli were transferred to a shoot differentiation and multiplication medium (A10 medium) with A4 medium as a control. These calli on A4 medium developed very slowly into few chlorophyllic regions between CA4 d30, CA4 d90 and CA4 d120 (**Figure 52c-e**, arrows). White nodular compact structures were also observed on calli CA4 d30 and could be considered as somatic embryos formation (**Figure 52c**, dashed arrow). Nevertheless, these nodular compact structures did not become chlorophyllic. By contrast, calli cultured on medium A10 became green at the tips after 30 days as observed on calli CA10 d30. These chlorophyllic tips could be

considered as shoot primordia (**Figure 52f**, arrows). The number and morphology of these chlorophyllic tips evolved rapidly and well–developed shoots could be seen on CA10 d60 calli (**Figure 52g**, arrows). One or two roots were perceived on these calli beyond 60 days of culture (**Figure 52h**). Rooting could probably enhance the multiplication, differentiation and elongation of shoots observed on CA10 d120 (**Figure 52i**). Three-cm size well developed shooty stems were excised and transferred onto the rooting medium (A5 medium) (**Figure 52j**) whereby roots were formed after two to three weeks.

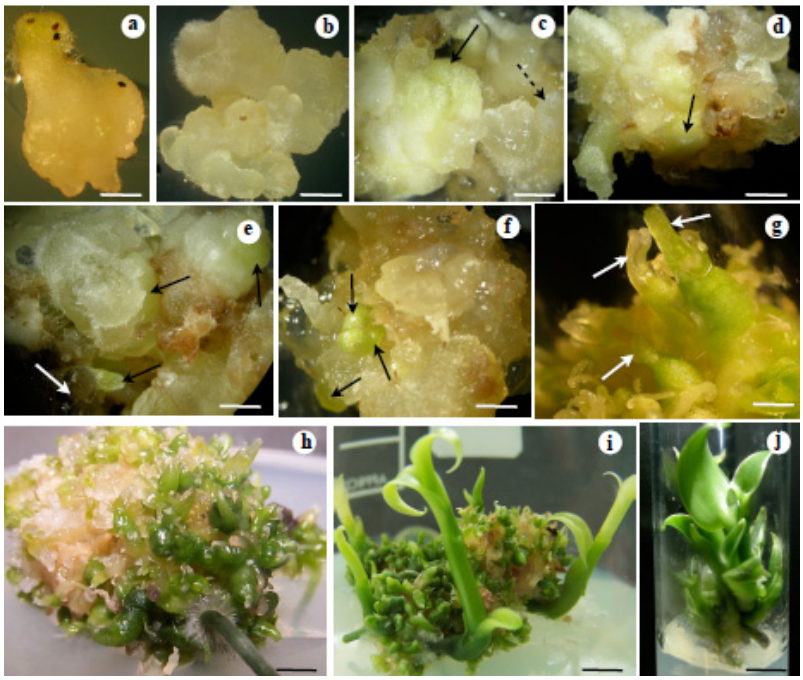

Figure 52: Plant regeneration from protocorm callus of *V. planifolia*. a. Development of protocorm-like structure (PLS) from seed on germination medium: BM + 0.5 mg l^{-1} TDZ; bar = 1.5 mm. **b.** Well-developed callus from PLS after transfer to callus induction medium: MB + 0.5 mg l^{-1} TDZ + 0.5 mg l^{-1} AIA; bar = 1 mm. **c.** Organogenic callus mass showing nodular compact structure (dashed arrow) and pale green zone (arrow) after 30 days culture on A4 medium: BM + 0.5 mg l^{-1} TDZ +0.3 mg l^{-1} AIA (i.e. CA4 d30 callus) ; bar = 750µm **d.** CA4 d90 callus with poorly-differentiated green structures (arrow) ; bar = 3mm. **e.** CA4 d120 callus exhibiting poorly-differentiated green structures (black arrows); necrotic area can be seen (white arrow); bar = 2.5 mm. **f.** Emerging shoot primordia (arrows) in 30-day-old organogenic callus on A10 medium: BM + 0.5 mg l^{-1}NAA (i.e. CA10 d30 callus); bar = 1 mm. **g.**CA10 d60 callus with buds formed from shoot primordia; bar = 350 µm. **h.** CA10 d90 callus exhibiting multiple shoot proliferation and rooting; bar = 3.5 mm. **i.** CA10 d120 callus exhibiting elongated shoots; bar = 8.5 mm. **j.** Rooted plantlet on A5 medium (BM) 15days after subculture; bar = 6.5 mm.

7.3.2 Metabolic profiling

Metabolic elucidation using one and two dimensional NMR spectroscopy

One- (1D) and two-dimensional (2D) NMR spectroscopy techniques are reliable methods for the analysis of a broad range of compound allowing the identification of compounds such as amino acids, carbohydrates, organic acids and phenolic compounds. 2D NMR spectroscopy, such as *J*-resolved, COSY (correlation spectroscopy) and HMBC (heteronuclear multiple bond correlation), was applied to identify metabolites from the congested ^{1}H-NMR signals. Thus, high-resolution NMR analysis has the potential to reveal the metabolic effect of enzymatic expression changes observed at proteomic level. **Figure 53** shows a ^{1}H-NMR spectrum of the metabolome of a 15-days-old protocorm derived calli sample. Signals at δ 5.40 (d, J = 3.8 Hz), δ 5.20 (d, J = 3.8 Hz), δ 4.59 (d, J = 7.9 Hz), δ 4.17 (d, J = 8.6 Hz) were assigned to the anomeric protons of the glucose moiety of sucrose, α-glucose, β-glucose and the fructose moiety of sucrose, respectively. Amino acids were identified at δ 2.82 (dd, J = 17.0, 8.2 Hz) and δ 2.95 (dd, J = 17.0, 4.0 Hz) as asparagine, at δ 2.13 (m) and δ 2.46 (m) as glutamine, at δ 1.48 (d, J = 7.2 Hz) as alanine, at δ 1.01 (d, J = 6.8 Hz) and δ 1.06 (d, J = 6.8 Hz) as valine. In addition to these compounds, γ-aminobutyric acid (GABA) was identified at δ 3.01 (t, J = 7.5 Hz), δ 2.30 (t, J = 7.5 Hz) and δ 1.90 (q, J = 7.5 Hz) (**Figure 53**).

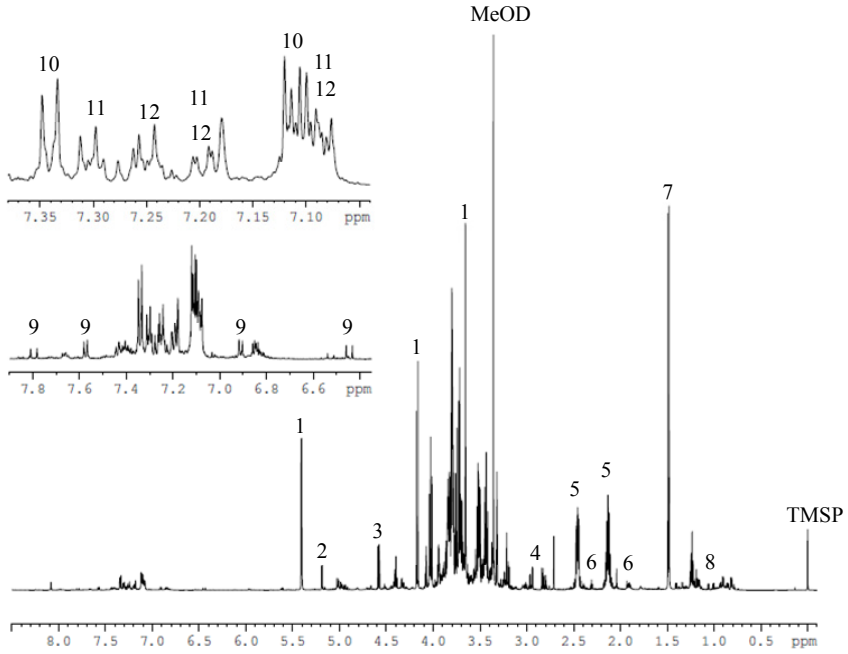

Figure 53: Typical ^{1}H-NMR spectra (CH_3OH-d_4–KH_2PO_4 in D_2O extract) of CA4 d15 callus in the range of δ 0 – 8.5, 6.5 – 7.9 and 7.0 – 7.4. Assignments: 1, sucrose; 2, α-glucose; 3, β-glucose; 4, asparagine; 5, glutamine; 6, γ-aminobutyric acid; 7, alanine; 8, valine; 9, *p*-coumaric acid; 10, *p*-hydroxybenzyl alcohol glucoside; 11, bis[4-(β-D-glucopyranosyloxy)-benzyl]-2-isopropyltartrate (glucoside A); 12, bis[4-(β-D-glucopyranosyloxy)-benzyl]-2-(2- butyl)tartrate (glucoside B).

In the aromatic region (δ 5.7-9.0), signals at δ 7.80 (d, *J* = 16.0 Hz), δ 7.58 (d, *J* = 9.8 Hz), δ 6.91 (d, *J* = 9.8 Hz) and δ 6.45 (d, *J* = 16.0 Hz) were assigned to p-coumaric acid; signals at δ 7.34 (d, *J* = 9.0 Hz), δ 7.11 (d, *J* = 9.0 Hz), δ 4.57 (s), δ 5.02 (d, *J* = 7.9 Hz) were assigned to *p*-hydroxybenzyl alcohol glucoside ; signals at δ 2.20 (m), δ 0.92 (d, *J* = 7.0 Hz), δ 0.86 (d, *J* = 7.0 Hz) were assigned to bis[4-(β-D-glucopyranosyloxy)-benzyl]-2-isopropyltartrate (glucoside A) and signals at δ 1.90 (m), δ 1.35 (m), 1.10 (m), δ 0.84 (d, *J* = 7.0 Hz), δ 0.77 (t, *J* = 15.0 Hz) were assigned to bis[4-(β-D-glucopyranosyloxy)-benzyl]-2-(2- butyl)tartrate (glucoside B) (**Chapter 3**). **Table 11** summarizes all compounds identified in ^{1}H-NMR spectra with the chemical shifts and the coupling constants of the signals.

Principal component analysis is an unsupervised clustering method requiring no knowledge of the data set and acts to reduce the dimensionality of multivariate data analysis while preserving most of the variance within it (Eriksson et al., 2006). The two first

principal components (PC1 and PC2) explained 63.9% of the variation in the entire dataset (**Figure 54**). Although PC1 discriminates CA4 d15 and CA10 d15 calli, the intra-group variation is unegligible compared to the inter-group one. Indeed, no qualitative differences between CA4 d15 and CA10 d15 calli were inferred from the visual inspection of the ^{1}H-NMR spectra. Furthermore, few quantitative differences were observed; as a result, there is not a strong separation between CA4 d15 and CA10 d15 calli in the PCA score plot (**Figure 54**). Nevertheless, as shown by the loading column plot of PC1, CA4 d15 calli were characterized by high content of sucrose, glucose and alanine while the other compounds detected were in a higher amount in CA10 d15 calli (**Figure 54**).

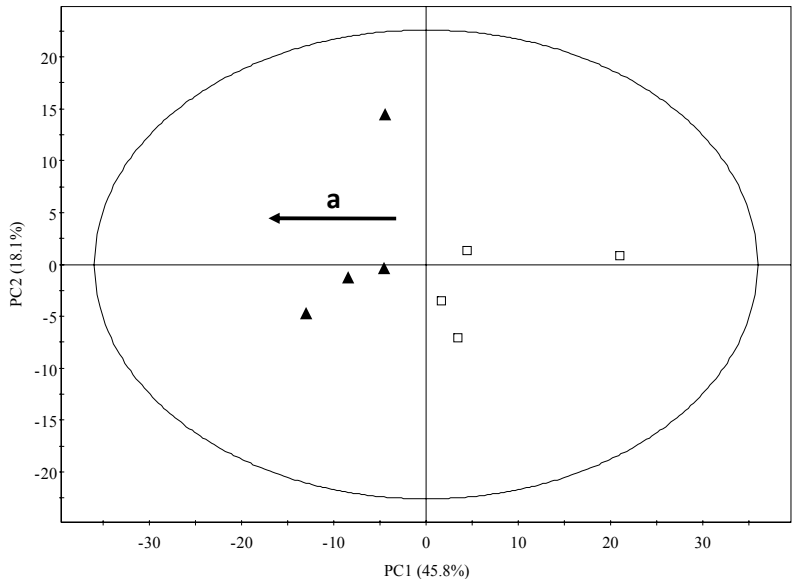

Figure 54: Score plot (PC1 vs. PC2) of PCA results obtained from ^{1}H-NMR spectra of CA4 d15 (▲) and CA10 d15 (□) calli; a: sucrose, glucose and asparagine.

Confirmation of phenolic compounds by LC-MS

A more targeted approach on phenolic compounds was performed by analyzing CA4 d15 and CA10 d15 calli samples using LC-MS. Presence of phenolic compounds that were identified by NMR has been confirmed with LC-MS by the detection of the ion mass of each compound.

Table 11: ^{1}H Chemical Shifts (δ), Coupling Constants (Hz) of CA4 d15 and CA10 d15 calli metabolites identified by references and using 1D and 2D NMR spectra (CH_3OH-d_4–KH_2PO_4 in D_2O, pH 6.0)

Compound	Chemical shifts and coupling constants
sucrose	δ 5.40 (H-1, d, *J* = 3.8 Hz), δ 4.17 (H-1', d, *J* = 8.6 Hz)
glucose	δ 5.20 (H-1, d, *J* = 3.8 Hz), δ 4.59 (H-1, d, *J* = 7.9 Hz)
asparagine	δ 2.82 (H-3a, dd, *J* = 17.0, 8.2 Hz), δ 2.95 (H-3b, dd, *J* = 17.0, 4.0 Hz)
glutamine	δ 2.13 (H-4, m), δ 2.46 (H-3, m)
γ-aminobutyric acid (GABA)	δ 3.01 (H-4, t, *J* = 7.5 Hz), δ 2.30 (H-2, t, *J* = 7.5 Hz), δ 1.90 (H-3, q, *J* = 7.5 Hz)
alanine	δ 1.48 (H-3, d, *J* = 7.2 Hz)
valine	δ 1.01 (H-4, d, *J* = 6.8 Hz), δ 1.06 (H-5, d, *J* = 6.8 Hz)
p-coumaric acid	δ 7.80 (H-7, d, *J* = 16.0 Hz), δ 7.58 (H-3, H-5, d, *J* = 9.8 Hz), δ 6.91 (H-2, H-6, d, *J* = 9.8 Hz), δ 6.45 (H-8, d, *J* = 16.0 Hz)
p-hydroxybenzyl alcohol glucoside	δ 7.34 (H-3, H-5, d, *J* = 9.0 Hz), δ 7.11 (H-2, H-6, d, *J* = 9.0 Hz), δ 4.57 (H-7, s), δ 5.02 (H-1', d, *J* = 7.9 Hz)
glucoside A	δ 2.20 (m), δ 0.92 (d, *J* = 7.0 Hz), δ 0.86 (d, *J* = 7.0 Hz)
glucoside B	δ 1.90 (m), δ 1.35 (m), 1.10 (m), δ 0.84 (d, *J* = 7.0 Hz), δ 0.77 (t, *J* = 15.0 Hz)

7.4 Discussion

In this work, the induction of shoot organogenesis from protocorm callus of *V. planifolia* was initiated for the first time. Using a metabolomics approach, biochemical and physiological markers at the origin of callus organogenesis were explored.

Metabolite analysis was conducted on the two calli samples (CA4 d15 calli and CA10 d15 calli) for four independent repeats of the biological experiment. In this study, sucrose, glucose and alanine contents were in a higher level in CA4 d15. Although the amounts of asparagine, glutamine, valine and phenolic compounds detected were higher in the shoot differentiation calli (CA10 d15 calli), none of these changes was statistically significant. Perhaps this is due to the small number of samples and/or the small metabolic variation between CA4 d15 and CA10 d15 calli. In addition, the calli cultures were stopped after 15 days at the stage where no visible differences between the CA4 and CA10 calli were observed; so CA10 calli were just at an early stage of the shoot differentiation and possible related metabolomic changes. This could explain the small difference observed between the two types of calli. Nevertheless, our results suggest that there is an increase in secondary metabolites (i.e. phenolic compounds) and a decrease in sugars (i.e. sucrose and glucose) in the early stages of shoot differentiation (**Figure 55**). Indeed, phenolic compounds are formed from the glycolysis via the shikimic acid pathway (Havkin-Frenkel et al., 1996; Mustafa and Verpoorte, 2007; Fortes et al., 2008). These metabolomic results are in accordance with those obtained from the proteomic analysis where enzymes from the glycolysis pathway were more expressed in the CA10 d15 calli as compared to the CA4 d15 calli (data not shown). The same is observed for amino acids and proteome changes such as the glutamine synthetase upregulation in CA10 d15 calli. Glutamine synthetase plays a major role in plant nitrogen metabolism (Miflin and Habash, 2002). Thus, among the amino acids involved in plant developmental process, GABA, asparagine and glutamine seem to accumulate in CA10 d15 calli as also observed by Bender et al. (1987), Murch et al. (1999), Dowlatabali et al. (2009). Interestingly, the phenolic compounds identified in the callus from *V. planifolia* are known as intermediates in the vanillin biosynthesis. Indeed, *p*-hydroxybenzylalcohol glucoside, bis[4-(β-D-glucopyranosyloxy)-benzyl]-2-isopropyltartrate (glucoside A) and bis[4-(β-D-glucopyranosyloxy)-benzyl]-2-(2-butyl)tartrate (glucoside B) were detected previously in *V. planifolia* green beans (Kanisawa et al., 1994; Palama et al., 2009). Another compound, the *p*-coumaric acid was also detected in the calli samples. Our results highlight the place of the *p*-coumaric acid as precursor in

the vanillin biosynthetic pathway (Podstolski et al., 2002; Havkin-Frenkel and Belanger, 2007). Furthermore, glucoside A and B were never reported previously in *V. planifolia* cell or tissue culture.

For this study, calli were obtained from protocorm of *V. planifolia* seeds. Inside the green pods, the accumulation area of glucovanillin was shown to be very close to the seeds (Odoux et al., 2003b). In these conditions, callus obtained from protocorm could be a material of choice for the study of the vanillin biosynthetic pathway. In our experiments, *p*-hydroxybenzylalcohol and glucosides A and B particularly are present in calli at early stage of shoot differentiation in CA10 calli. This could mean that those compounds in *V. planifolia* are accumulated in the younger or photosynthetic tissues (i.e. embryos or leaves). Theses compounds could then be transported to developing pods and later converted to glucovanillin which concentration has been previously shown to increase in the developing pods (Kanisawa et al., 1994; Palama et al., 2009).

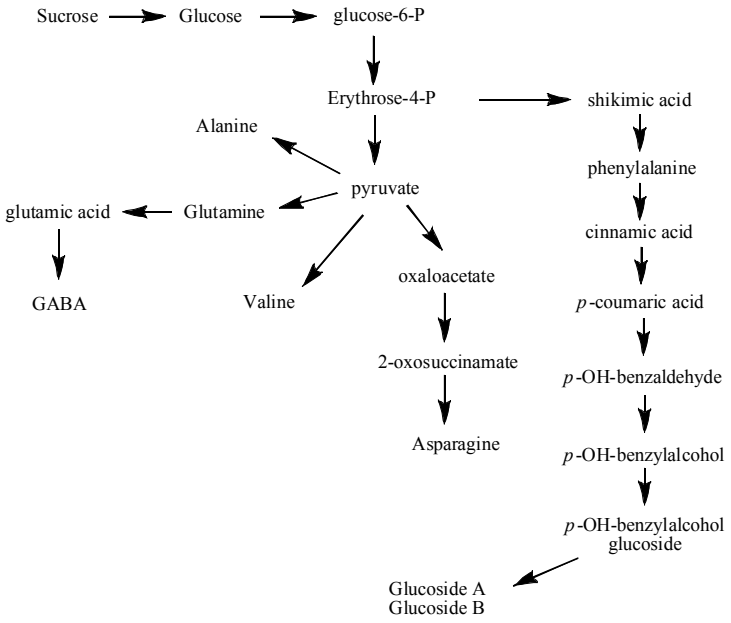

Figure 55: Proposed metabolic pathway in *V. planifolia* calli based on the metabolites identified and general plant biosynthesis.

7.5 Conclusion

In this study, we investigated the effects of plant hormones on the biochemical mechanisms that release shoot formation during *V. planifolia* callus differentiation. The cellular reprogramming in this morphogenesis process suggested an early stimulation of several metabolic pathways including mobilization of sucrose, glycolysis and phenolic compound synthesis, amino acids synthesis among others to assemble the photosynthetic machinery in the cells. Metabolomic analysis showed that at a very early stage of plant development coumaric acid and glucoside A and B are already produced. Since these compounds are precursors of vanillin, callus samples could be a plant material to study the biosynthesis of vanillin precursors and further studies should be of interest.