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Sugar metabolism of *Vetiveria zizanioides* cells cultured with Amberlite XAD-4. Uptake of radiolabelled sugars, cell wall invertase activity and gene expression

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Abstract: Activated and non-activated Amberlite XAD-4 was added to the cell cultures of vetiver (*Vetiveria zizanioides* Stapf.) in order to study its effects as abiotic growth promoter. Amberlite XAD-4 increased vetiver cell viability, especially when used in the activated form. Results from labelling experiments in cell suspensions showed that XAD-4 prompted a significant decrease of sucrose Km, accompanied by an increased Vmax. Glucose uptake from the medium was almost doubled, as demonstrated by the increase of the radiolabelled fraction (25-fold the controls) in vetiver cells cultured with XAD-4. Affinity for fructose uptake by cells was not altered by the use of activated XAD-4, even though its uptake and Vmax increased. These results were further supported by a significant 7.5-fold increase in cell wall invertases (CWI) activity in XAD-4 elicited cells. Vetiver CWI gene expression was characterized by transcript RT-PCR analysis. The identity of the transcripts was confirmed by sequencing analysis. These results indicated the use of XAD-4 as an effective tool for vetiver cell biomass optimization.

Abbreviations

CWI	cell wall invertases (β -fructofuranosidases; EC 3.2.1.26)
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
FDA	fluorescein diacetate
PVP	polyvinylpyrrolidone

Introduction

Vetiveria zizanioides Stapf. is a graminaceous plant native of India, renewed for the essential oil extracted from the root. Vetiver essential oil contains more than 150 sesquiterpenes (Maffei 2002), whose biosynthesis has been reviewed (Akhila and Rani 2002). Vetiver plants produce short-lived seeds and are normally propagated vegetatively. Given the impossibility to obtain novel cultivars by conventional breeding methods, plant regeneration via tissue cultures can be conveniently exploited. The final focus is the selection of somaclonal variants with more oil or with a different oil composition (Mucciarelli and Leupin 2002). Plants that are regenerated starting from dedifferentiated callus and cell suspensions, normally allow for more variation to be obtained (Mathur et al. 1989) and, in turn, can be exploited for in vitro biotransformation experiments (Mucciarelli and Leupin 2002; Del Giudice et al. 2008). In vetiver, however, plant regeneration has been obtained almost exclusively from leaf explants (Mucciarelli et al. 1993; Leupin et al. 2000) and protocols for the rapid establishment of cells in liquid media starting from vetiver roots are still unsuitable. To date, experiments aimed to manipulate cell culture environment of vetiver cells in order to induce new morphogenetic patterns of growth and elicit essential oil biosynthesis have not yet been tried.

An important nutritional factor controlling in vitro cell growth and morphogenesis is represented by the sugar moiety of culture media (Choi et al. 2008; Cloutier et al. 2008). Sugars as the main carbon source for non-photosynthetic cells are utilized to maintain most of the biological and physiological activities controlling the synthesis and release of secondary products into the culture media (Lamboursain and Jolicoeur 2005).

Many plant cells in culture commonly utilize sucrose, glucose and fructose and some other monosaccharides equally well when they are added to the culture medium as a sole carbon source. However, some plants have preferential requirements for glucose or sucrose (Kato et al. 2007). Sucrose administered externally to plant cell suspensions is usually rapidly hydrolyzed to glucose and fructose by cell wall invertases (β -fructofuranosidases; EC 3.2.1.26) (Goetz and Roitsch 2000; Kretschmar et al. 2007). The role of invertases in sucrose uptake is regarded as prominent in sink tissues in which there is no symplastic continuity between cells (i.e. maternal and embryo tissues of developing seeds) (Jain et al. 2008; Privat et al. 2008), and in the case of cultured cells (cell suspensions and calluses) where cell wall connections are very loose (Koch 2004; Kretschmar et al. 2007). In the case of cultured tissues, however, invertases may be secreted into the medium also (Shin et al. 2003) and a novel isoform of acid invertase, which is not bound to the cell wall, has been characterized (Kim et al. 2000). Depending on the species and cell type, hexoses released into the culture media are then taken up by passive or active processes, through hexose-specific transporters (Stepan-Sarkissian and Fowler 1986; Roitsch et al. 2003; Kato et al. 2007). Once inside, hexoses have a great capacity to stimulate specific sugar sensors (Koch 2004 and references therein) and the resulting signals can alter expression of diverse genes (Smeekens 2000; Rolland et al. 2002; Sherson et al. 2003). With their hydrolytic activity, therefore, invertases are potentially strong effectors of widely varying developmental processes (Tang et al. 1999; LeClere et al. 2008), including the biosynthesis and perception of auxins and abscisic acid (Koch

2004 and references therein; Arru et al. 2008). Hormone sensing by cultured cells is a prerequisite for cell growth, which has restricted in the past a wider application of plant cell cultures (Chang and Sim 1995). This is the case for many crop cereals and other economically important grasses like vetiver. The addition of a liquid or solid phase to culture media has proven to be effective in maintaining cell viability over a long period of culture, with the advantage of enhancing the production and release of plant secondary metabolites from cells (Strobel et al. 1991; Yan et al. 2005) and physiological rationale for this has never been elucidated.

Preliminary results from vetiver cell suspensions, have demonstrated that cell growth and viability can be triggered by the addition to the medium of the non-ionic polymeric adsorbent Amberlite XAD-4 (Mucciarelli et al. 1994). With the aim to establish a protocol for good quality biomass production, here we elucidate some aspects of sugar uptake in vetiver cell suspensions under the application of Amberlite XAD-4. By means of radiolabelled sugar analysis, the observed stimulation of acid invertase activity and related CWI gene expression has been interpreted.

Materials and methods

Plant material

Vetiveria zizanioides Stapf. callus cultures were initiated from internode segments of plants growing in experimental plots of the Department of Plant Biology (Maffei et al. 1995) and maintained on a MS medium (Murashige and Skoog 1962) modified according to Mucciarelli and colleagues (Mucciarelli et al. 1993).

Cell suspensions

Callus cultures in liquid media were established by transferring 6 g (FW) of callus in 80 ml of MS medium. Suspension cultures were maintained on a gyrotary shaker at 25 rpm for 30 days. All in vitro cultures were carried out in a growth chamber (26°/28°C day, 21°C night, with 14-h photoperiod, 37 $\mu\text{mol m}^{-2} \text{s}^{-1}$ Fluorescent TLD Philips lamps). Cell culture viability was tested with fluorescein diacetate (Widholm 1972). Viability values are expressed on a percent basis.

Amberlite XAD-4 resin

Amberlite XAD-4, purchased from Sigma, has been used in activated and non-activated form according to Loomis et al. (1979) and added after sterilization (120°C, 1 atm, 20 min) at 2% (w/v) to culture media. Table 1 lists the chemical-physical characteristics and properties of Amberlite XAD-4 resin used in this work.

Resin Type	Chemical nature	Density (g ml ⁻¹)	Surface (g ² ml ⁻¹)	Mesh size	Pore diam. (Å)	Properties
XAD-4	Styrene–divinyl–benzene copolymer hydrophobic is adsorbent	1.08	725	20–60	50	Product enhancement and recovery from cell cultures; found to be the best polymeric adsorbent for removing phenolic compounds from aqueous streams (Li et al. 2001)

Table 1 Chemical-physical characteristics and properties of Amberlite XAD-4 resin used in this work.

Determination of sugars in culture media

Sugar concentrations of culture media were determined with fractionating extraction and enzymatic hydrolysis and analyzed by gas chromatography and mass spectrometry (GC-MS). Thirty days after inoculum, cells were filtered and liquid media extracted in chloroform with the formation of two phases: the aqueous one was evaporated under vacuum at 60°C and then re-suspended in 80% methanol and concentrated again by the same procedure. The chloroform phase was evaporated under vacuum at 50°C. Methanolic extracts were separated by column chromatography by using Polyclar AT in order to bind phenolic compounds (Loomis and Battaile 1966). An increasing gradient of methanol/water was applied from 60% up to 100% of methanol.

Aqueous extract was concentrated and the methanolic one hydrolyzed by β -glucosidases (Sigma) according to Mabry and colleagues (1970). After enzymatic hydrolysis, samples were passed on a Polyclar AT column as above. Sugar extracts were then resuspended in anhydrous pyridine and derivatized by using 1,1,1,3,3,3-hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) according to Creaser and colleagues (1989).

GC-analysis was conducted in a HP-6890 by using an HP-5 column (25 m×0.2 mm×0.33 μ m). Analysis conditions: initial temp. 100°C for 5 min, then a linear increase of 4°C per min up to a 290°C final temperature maintained for 5 min. Injection temperature 270°C, FID temperature 290°C. Compound identification was performed by means of gas chromatography-mass spectrometry as previously described (Maffei et al. 1999).

Radiolabelling experiments

Uptake experiments were conducted on cell cultures established as above. Cell aliquots were suspended with 0.5 mM 2-[N-Morpholino]ethanesulfonic acid sodium salt (MESNaOH) buffer, pH 5.70, containing 0.125 mM K₂SO₄ and 0.5 mM CaSO₄ to facilitate sugar absorption. Immediately after, 0.1 mM to 50 mM [U-¹⁴C]-sucrose, [U-¹⁴C]-D-glucose and [U-¹⁴C]-fructose (Amersham Pharmacia, UK; specific activity 565 mCi mmol⁻¹, 310 mCi mmol⁻¹ and 289 mCi mmol⁻¹, respectively) were added to each samples and incubated for 1–30 min. Each sample was then rapidly filtered through a 45 μ m mesh Millipore filter and washed quickly twice with MES-NaOH

buffer. Cells were extracted overnight in 80% ethanol, at 50°C. Washing solutions were immediately analyzed. All analyses were conducted in a Packard 1500 TRI-CARB liquid scintillation analyzer with Pico-Fluor 40 as a quencher. The apparent Michaelis constant (K_m) and the apparent V_{max} for sucrose, fructose and glucose were calculated from a Lineweaver-Burk plot.

Cell wall invertase extraction

All operations were carried out at 4°C. Thirty days after inoculum, vetiver cells were filtered and used for enzyme extraction and assay. The material was ground in liquid nitrogen by mortar and pestle in five volumes of cold 50 mM KH_2PO_4 , pH 7.2, containing 0.1 mM DTT, 0.1 mM EDTA, 10 mM Na_2SO_3 and 1% (w/v) PVP. The final homogenate was squeezed through a cheesecloth, homogenized in a tight-fitting chilled Ten-Broeck homogenizer and centrifuged at 15,000 g for 30 min at 4°C. The pellet was suspended in 50 ml sodium acetate, pH 4.5, and stirred overnight at 4°C. After centrifugation, the pellet was resuspended in a minimum volume of 50 mM sodium acetate, pH 4.5, and employed directly for protein assay and enzyme activity.

Cell wall invertase assay

Cell wall invertase activity was recorded spectrophotometrically, in a coupled reaction, following $NADP^+$ reduction at 340 nm in the presence of an excess of hexokinase and glucose 6-phosphate dehydrogenase. 100 μ l of crude extracts were incubated at 28°C for 30 min in 250 μ l of acetate buffer in the presence of 1 M sucrose. The reaction was stopped by heat denaturation at 80°C for 3 min and cooled down at room temperature. Fifty mM Tris-HCl, pH 7.6, containing 2.5 mM ATP, 3.0 mM $MgSO_4$, and 0.36 mM $NADP^+$ was added to the mixture. The reaction was started with hexokinase/glucose-6-phosphate dehydrogenase (6U/3U).

Protein determination

The protein content of the extracts was determined according to Bradford (1976) and using bovine serum albumin as standard protein. Specific activity of the cell wall invertase is reported as μ Kat $mg\ prot^{-1}$.

Total RNA extraction and RT-PCR assays

Total RNA for reverse transcriptase (RT) PCR analyses was isolated from cells grown in the presence or absence of activated XAD-4 according to Viotti and colleagues (1982). Reverse transcription was performed at 42°C for 60 min in 20 μ l of a reaction mixture containing 1 \times Reverse Transcriptase buffer, 0.2 mM DTT, 1.0 mM dNTPs, 0.25 μ g of oligo(dT) 21-mer, 200 U of M-MLV Reverse Transcriptase (Sigma) and 1 μ g of total RNA. Two microliters of reverse transcription product were amplified in a 50 μ l PCR reaction mixture containing 1 \times PCR buffer, 25 μ M forward primer (5'-CTCATGTGCACCGACCCAC-3') and reverse primer (5'-GCGCCGAAGCTCTCGACCAC), 1U Taq DNA polymerase (Amersham Pharmacia Biotech), 0.2 mM dNTPs. The PCR parameters were as follows: 94°C for 2 min; 94°C for 1 min, 56°C (annealing temperature) for 1.5 min and 72°C for 1 min for 35 cycles; and a final extension at 72°C for 7 min. The pair of primers employed in the PCR reaction correspond to nucleotides 1476 to 1496 and

1602 to 1,622 respectively, in the sequence of maize Incw1 cDNA (Taliercio et al. 1999). An equal amount of PCR products were separated by electrophoresis in 1.2% agarose gels and detected by staining with ethidium bromide. An amplified band of about 150 bp was gel purified using GFXM PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech) and used directly for sequencing with the same primers employed in the PCR reaction.

Sequence analysis

Sequencing was done using the ABI 310 capillary sequencer according to the manufacturer instructions (ABI System, Perkin Elmer). Sequence comparison against the Genbank non-redundant protein database was performed by using the BLASTX algorithm (Altschul et al. 1997). Following sequence analysis was conducted using ClustalX program and GeneDoc programs.

Statistics

At least three replicates were performed for each experiment. Significance of treatment effects were determined by using analysis of variance using Systat 5.2 software for Macintosh. The Tukey-Kramer HSD was used for post hoc comparison. Mean values were reported along with their standard errors (\pm).

Results

Cell viability of vetiver control cultures was rather low since the beginning of the inoculum (data not shown) and reached 40–50% of viable cells by eight days of culture (Figure 1). At 28 days most cells clumped together into small and big aggregates and resulted FDA negative (Figure 1).

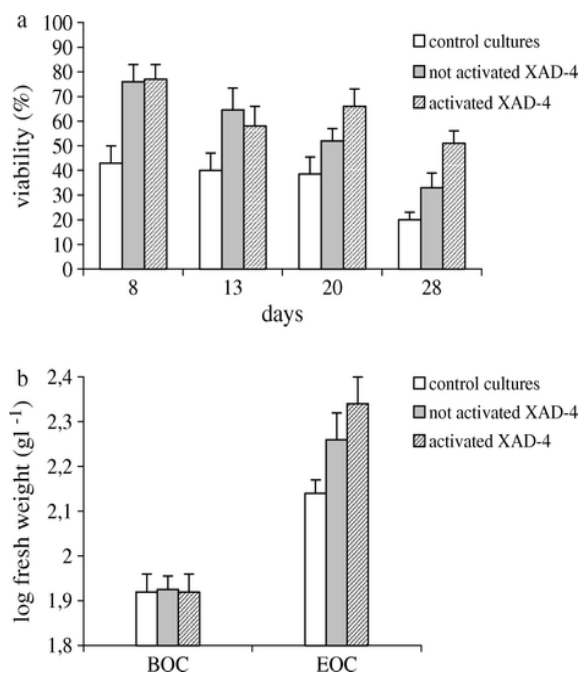


Figure 1. Viability and cell growth of *Vetiveria zizanioides* cell suspensions with amberlite XAD-4 resin. (a) Comparison between controls and XAD-4 cell viability; resin added in activated and non-activated form. (b) Comparison between final fresh weights of control and XAD-4 vetiver cultures. BOC: total cell biomass at beginning of culture; EOC: total cell biomass at the end of culture (30th day). Bars indicate standard errors.

After eight days of culture with Amberlite XAD-4, almost 81% of cells were viable with both the activated and not-activated resin (Figure 1). By the following two sampling periods, XAD-4 supplemented media proved to be superior to the controls, even if mean differences in cell viability between the two XAD-4 treatments were not significantly different. By the end of culture, the number of FDA positive cells dropped to 51% and 34% in activated and non-activated XAD-4 media, respectively, when most of the cells were dead in control cultures (Figure 1). Vetiver cell biomass was increased significantly by the resin, but no significant differences between activated and non-activated XAD-4 were found (Figure 1).

Table 2 shows the total content of sucrose, glucose and fructose before cell inoculation (i.e., the content of the medium after incubation) and at the end of the culture period in XAD-4 (activated and non-activated) cultures and in the control (cells grown without the resin). Table 2 also shows the percentages of sugar consumption with respect to total sugar content without cell inoculation.

Specifications	Sucrose	Glucose	Fructose
Medium	19.90 ^a (100)	16.14 ^a (100)	1.29 ^a (100)
Control culture	0.06 ^b (-99.70)	13.96 ^b (-13.50)	2.17 ^b (+68.22)
Resin (activated)	0.00 ^b (-100)	6.81 ^c (-57.87)	0.21 ^c (-83.72)
Resin (not activated)	2.24 ^c (-88.74)	5.51 ^d (-65.86)	0.79 ^d (-38.76)

Values are expressed as mg ml⁻¹. Percentage values are indicated on brackets. Negative values indicate the percentage of consumption with respect to the medium content (assumed as 100%), positive values indicate the percentage of increase in the medium. In the same columns, same letters indicate not significant differences; different letters indicate significant ($p < 0.05$) differences.

Table 2 Total sucrose, glucose and fructose content of the culture medium before inoculum (medium) of *Vetiveria zizanioides* cells, in control cell cultures at the end of the culturing period (30th day) and in the same after the addition of activated and non-activated XAD-4.

In control cultures, sucrose was almost completely used by cells, whereas the content of glucose was still high (13.5% consumed) and that of fructose increased (Table 2).

In the presence of activated XAD-4 resin, sucrose was also completely consumed, the use of glucose increased (four-fold consumption) and fructose was significantly depleted from the medium (83.7% consumption). Also with non-activated XAD-4 resin a significantly decrease of glucose and fructose levels in the medium was registered, but this corresponded to a significant reduction of sucrose consumption. In non-activated XAD-4 the consumption of fructose was lower and that of glucose was higher, when compared to that activated resin (Table 2).

In order to evaluate the kinetics of sugar uptake, labelled sucrose, fructose and glucose were fed to *V. zizanioides* cell cultures after a starvation period of 12 h in the presence of 0.5 mM CaSO₄. Analysis of uptake kinetic for sucrose, glucose and fructose was done in control cultures and in the presence of the activated resin. Results indicated that in control cultures, glucose has the major affinity, followed by fructose and sucrose whereas in the presence of activated XAD-4, sucrose has the major affinity (Table 3). In the controls, the uptake rate for the three labelled sugars was higher for fructose, which was followed by glucose and sucrose. In the presence of activated XAD-4, glucose uptake was consistently increased, and almost 25-fold higher than in control cultures.

Specifications	Sucrose	Glucose	Fructose
Control culture			
<i>K_m</i> (mM)	3.5 ^a	1.9 ^a	2.5 ^a
<i>V_{max}</i> (nmol h ⁻¹ kg ⁻¹)	12.0 ^b	45.0 ^b	65.0 ^b
Activated XAD-4			
<i>K_m</i> (mM)	1.6 ^c	4.1 ^c	2.1 ^a
<i>V_{max}</i> (nmol h ⁻¹ kg ⁻¹)	27.0 ^d	1150.0 ^d	139.0 ^c

In the same columns, same letters indicate not significant differences; different letters indicate significant ($p < 0.05$) differences.

Table 3 *K_m* and *V_{max}* values for sucrose, glucose and fructose after feeding *Vetiveria zizanioides* cell cultures with [U-14C]-sucrose, [U-14C]-D-glucose and [U-14C]-fructose in the presence of activated XAD-4 and in the controls.

Fructose and sucrose uptake was also significantly higher in the presence of activated XAD-4 (Table 3). Cell wall invertase activity was affected by the presence of the activated XAD-4 resin. The activity of the cell wall invertase detected in cell suspensions incubated with XAD-4 was 1.67 (± 0.02) μ Kat mg⁻¹ protein, whereas the activity recorded in the control cells was 0.22 (± 0.01) μ Kat mg⁻¹ protein (Figure 2). The presence of the activated resin caused a 7.5-fold increase in enzyme activity.

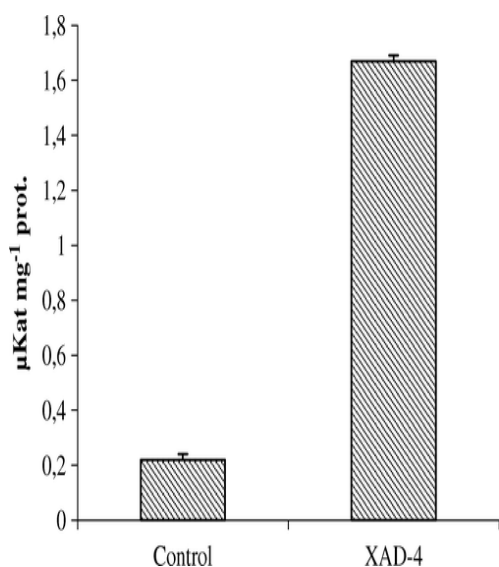


Figure 2 Cell wall invertase activity in *V. zizanioides* cell suspension. Enzyme activity is expressed in $\mu\text{Kat mg prot}^{-1}$.

In order to see if this increase in enzyme activity was correlated with a different level of gene expression, total RNA was extracted from vetiver cells grown with and without Amberlite XAD-4. Cell wall invertase expression was characterized by monitoring transcript accumulation using RT-PCR analysis. A pair of primers was designed from maize *Incw1* sequence, and PCR was performed using the first-strand cDNA as a template. An amplified band of about 150 bp was detected in cell suspension grown in the presence of Amberlite XAD-4 resin (Figure 3, lane 3), while only a faint band was visible in the control cells (Figure 3, lane 2). No PCR products were amplified without reverse transcription reactions (Figure 3, lane 4).

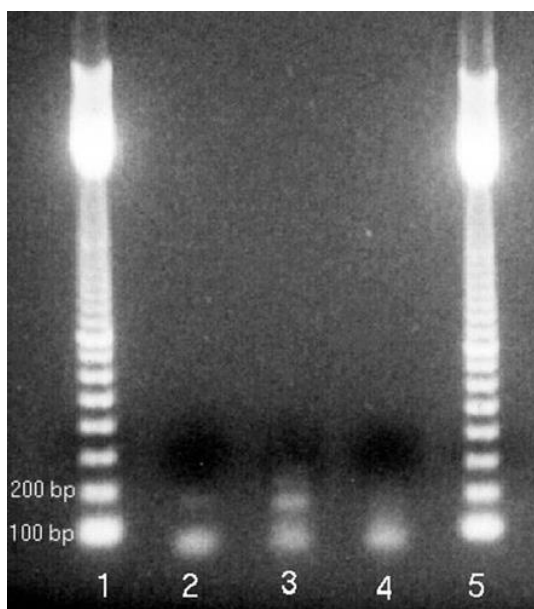


Figure 3 Changes in the presence of the cell wall invertase transcript in *V. zizanioides* cell suspension grown without or with Amberlite XAD-4 resin, as determined by RT-PCR analysis. Lane 1 and 5: 100 base-pair ladder (Amersham Pharmacia Biotech); lane 2: RT-PCR of RNAs extracted from control cell suspension; lane 3: RT-PCR of RNAs extracted from cell suspension grown in the presence of Amberlite XAD-4 resin; lane 4: PCR of the same RNAs without RT (negative control).

The sequence analysis of the 150 bp band, excised and gel purified, confirmed that the cDNA product was amplified from the transcribed product of *V. zizanioides* gene. Analysis of the predicted amino acid sequence of *V. zizanioides* CWI revealed that it possesses some features reported for other CWIs (Figure 4). The deduced amino acid sequence of this cDNA fragment showed 44% identities with the corresponding region of maize Incw1 (Figure 4), of *Saccharum officinarum* and *Oryza sativa*, whereas it showed a 40% identities with the maize Incw2. On the basis of these results, we identified this as a partial sequence of putative *V. zizanioides* CWI.

	*	20	*	40	
ZmIncw1 :	CTDPTKSSLS	PDLYKPTLAGLVDADI	SSGKIILRSLID	--RSV	ESF : 539
OsCIN1 :	CTDPTKSSLS	PDLYKPTFAGFVDDI	SSGKISLRSLID	--RSV	ESF : 525
ZmIncw2 :	CTDPTKSSLN	PNLYRPTFAGFVDDI	SSGKISLRSLID	--RSV	ESF : 541
Shcw1 :	CTDPTKSSLS	PDLYKPTFAGFVDDI	SSGKIILRSLID	--RSV	ESF : 477
VzCWI :	CTSP---	SLPPDPIEPTVSSI	LDSDLRSGKVP	LKSLTSDHR	SVYESF : 44

Figure 4 Amino acid sequence comparison of *V. zizanioides* cell wall invertase fragment (VzCWI) to the corresponding regions of other cell wall invertases isolated from the grass family. *Oryza sativa* (cultivar japonica-group) (OsCIN1, accession no. AB073749; Hirose et al. 2002); *Zea mais* Incw1 (Zmlcw1, accession no. AF050129; Taliercio et al. 1999); *Zea mais* (Zmlcw2, accession no. AF050128 Taliercio et al. 1999); *Saccharum officinarum* (Shcw1, accession no. AY302084; Peters et al. unpublished). The alignment was created using the ClustalX program. Shading indicates conserved identity for the aligned amino acids: black background shading indicates 100% conservation, dark grey shading indicates 80% conservation, and light grey shading indicates 60% conservation. Asterisks indicate residues that are highly conserved or absolutely conserved among the grass family cell wall invertases examined.

Discussion

Most grasses, including many economically important cereal crops, show both strict genotype dependence and recalcitrance to *in vitro* culture and plant regeneration. In *V. zizanioides* this was the case when attempting to obtain somaclonal variants for essential oil production in cell cultures.

As shown in the paper of Leupin et al. (2000) on vetiver cell cultures, several growth parameters among which plant regulators, incubation time and sucrose concentration, were essential to obtain high percentages of regenerates. Very high sucrose levels (75 g l⁻¹) in particular, were found to elicit embryogenesis and plant regeneration at the end of the culture (Leupin et al. 2000).

Data presented in this work (Table 2) showed that after 28 days of incubation, vetiver cells (control cultures) had already consumed almost all the sucrose of the culture medium, while glucose and fructose concentrations were still very high. This finding supports Leupin et al.'s (2000) results on better regeneration efficiency of vetiver through an increase of sucrose concentration into the media. The high amount of sucrose in the media could compensate for low efficiency in sucrose uptake by vetiver cells.

Our results showed that sucrose in the medium was rapidly hydrolyzed extracellularly to glucose and fructose, and both accumulated into the medium being probably little utilized by vetiver cells. In the suspension cultures of many plant species, sucrose hydrolysis is brought about by cell-wall-

bound invertases (CWI) (Sturm and Tang 1999; Xiaoli et al. 2000 and references therein). The following import of hexoses into plant cells depends on the presence of hexose-specific transporters in the plasma membrane.

In this report we described how the use of XAD-4 resin in culture media improved vetiver growth and viability, both through a significant better uptake of sucrose and a faster consumption of the glucose present in the medium.

At the end of the culturing period with XAD-4, sucrose was completely consumed as for controls (Table 2), but the resin induced also a noticeable decrease of glucose and fructose concentrations. Differential affinity for glucose and fructose has been reported for many plant cell cultures (Sherson et al. 2003; Kretzschmar et al. 2007 and references therein). These monosaccharides represent the principal source of carbon and energy in most cells, and a significantly higher affinity of the membrane transporters for glucose is almost the rule.

Our data suggested that the addition of the polymeric adsorbent XAD-4 to the culture medium of vetiver resulted in a significant increase of cell wall invertase activity (7.5-fold higher with respect to the controls), and this might have improved sugar hydrolysis into the media through a faster enzyme activation. This higher CWI activity in the presence of XAD-4 was paralleled by an increased expression of the corresponding gene as it was revealed by RT-PCR experiments.

Shin et al. (2003) have demonstrated that CWI plays a crucial role in the control of metabolic fluxes in red beet liquid cultures, with patterns of sugar uptake similar to those observed in vetiver cells. When sucrose is added to the medium, it can be taken up by cells either via monosaccharide transporters after extracellular sucrose hydrolysis (cell wall-bound invertases) or directly via plasma membrane-localized, sink-specific sucrose transporters (Büttner and Sauer 2000 and references therein).

One of the defining questions in plant cell culture, however, is understanding how cells uptake nutrients from the culturing media. Besides nitrogen and other nutrients, sugars are thought to be the most important sources for cell growth (Bush 1999). Results from labelling experiments showed that *V. zizanioides* cell suspensions are able to uptake [U-14C]-sucrose, [U-14C]-D-glucose and [U-14C]-fructose. The kinetics of sugar uptake into plant cells is frequently described as being biphasic, with a saturable component linked to an active transporter functioning at concentrations of a few millimolar, and a non-saturable component linked to a facilitated diffusion at higher concentrations (Stanzel et al. 1988; Bouteau et al. 1999). In our experiment sucrose, glucose and fructose showed a biphasic uptake curve (data not shown); in the range of 0–2 mM, uptake characteristics fitted active uptake via a carrier, showing saturation (Michaelis-Menten) kinetics with values typical of sugar uptake in plant cell cultures (Rausch 1991; Botha and O'Kennedy 1998; Krook et al. 2000).

The effect of activated XAD-4 was a significant increase in sucrose affinity, which was accompanied by an increased V_{max} . These data explained the almost complete depletion of sucrose observed in the liquid medium and suggest both an increased activity and number of sucrose transporters.

Glucose consumption in the medium was almost doubled by activated XAD-4 (Table 2), while radio-labelled glucose inside vetiver cells increased about 25-fold, with respect to controls. These data suggested an increased activity of both glucose transporters and cytosolic/vacuolar invertase activity (Weber and Roitsch 2000).

Although fructose is present at low levels in the intracellular and extracellular compartments of plant cells, no transporter specific for fructose has yet been identified (Delrot et al. 2000). In *Daucus carota* cell suspensions, fructose and glucose uptake is both a passive and active transport by carriers (Krook et al 2000). In *V. zizanioides*, affinity for fructose uptake was not altered by the use of activated XAD-4 even though the resin stimulated the hexose consumption and increased V_{max} .

The results of this work indicated that Amberlite XAD-4 promotes cell growth of *V. zizanioides* cell suspensions and this was accompanied by an improvement of sugar uptake from media. The uptake of sucrose, despite the increased expression and activity of the CWI, suggests that sucrose hydrolysis, though important, is not essential for sugar assimilation in *V. zizanioides* cell cultures (in accordance with Lemoine et al. 1988; Shin et al. 2003).

XAD resins adsorb organic matter mainly by hydrophobic bonding, even though the exact mechanism of adsorption is still unknown (Lepane 1999). They are widely used in several applications: from binding of proteins to heavy metals pre-concentration, and from sequestration and detoxification of toxic organic compounds to adsorption of secondary metabolites from plant cell and tissue cultures (Chang and Sim 1995; Peuravuori et al. 1997; Kabay et al. 1998; Li et al. 2001; Uzun et al. 2001; Choi et al. 2005; Pavlov et al. 2005). Amberlite XAD-4 copolymer is found to be the best polymeric adsorbent for removing phenolic compounds from aqueous streams (Li et al. 2001; Ming et al. 2006). A detoxifying action of the resin against both chemical impurities of the growing medium and cell released metabolites cannot be excluded (Green and Thomas 1996) as well as a direct action of XAD-resin towards cell primary metabolism.

Our study suggests reconsidering the nature of carbon sources to be added to nutritional media for cell cultivation and indicated XAD-4 addition to the media as an effective tool for cell biomass optimization.

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