Pak. J. Bot., 46(6): 2195-2202, 2014.

DEVELOPMENT OF A VINASSE CULTURE MEDIUM FOR PLANT TISSUE CULTURE

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Abstract

Vinasse is the main pollutant (effluent) obtained from the distillation of sugarcane in the production of fuel alcohol. However, this residue is rich in nutrients that are required by plants. We developed a new culture medium using vinasse for the *In vitro* propagation of an orchid. The vinasse was treated (decanted and filtered), and the nutrients were determined and quantified. Different formulations using vinasse were tested for an *In vitro* culture. The vinasse dilutions demonstrated a good buffering effect. The ideal vinasse dilution for media formulation was 2.5%. The best KC formulations with vinasse were KCV1 and KCV5. Compared to KC medium, these formulations demonstrated similar results for *In vitro* multiplication, with the exception of protocorm-like body number, which was inferior in the vinasse formulations. Conversely, for *In vitro* elongation and rooting, these vinasse media were superior to KC medium. KC medium promotes a low rooting rate (8%) compared to 68 and 100% obtained by KCV1 and KCV5, respectively. Moreover, plantlets cultured on KC medium become protocorm-like body clusters, which impeded the acclimatization of these explants. Plantlets elongated and rooted on KCV1 and KCV5 were successfully acclimatized with a 91% survival rate for both KC vinasse formulations. This study shows the great potential of this technology as a rational alternative to vinasse disposal and adds value to what is currently considered a waste product.

Key words: Orchid; Biofactories; Micropropagation; Vinasse disposal; Vinasse composition; BOD; COD; *Oncidium*.

Introduction

Anthropogenic activities resulted in the production of more wastewater thus becoming matter of serious concern (Ahmad *et al*., 2013). Vinasse is the main effluent obtained from the distillation of the fermented sugarcane juices used to produce fuel alcohol and presents high pollutant potential. For each liter of fuel alcohol produced, approximately 10 to 18 liters of vinasse are produced, depending of the technological level of the distillery (Da Silva *et al*., 2007). The large volume of this residue that is produced, billions of liters per year, is of a great environmental concern. Vinasse disposal has a great environmental impact on lakes, rivers, streams and underground waters due to high BOD (biochemical oxygen demand) and COD (chemical oxygen demand), which results in the suffocation of aquatic fauna and flora, resulting in the death of aquatic life. However, this residue is rich in several mineral nutrients that are required for plant growth, and its application on crop fields increases yield (Almeida, 1952). Nevertheless, the constant application of vinasse in excess on the soil increases soil salinity due to the high content of potassium and therefore, this practice is prohibited in several places.

Tissue culture constitutes a way to maintain available competent explants (plant parts) free from contamination for use in *In vitro* propagation (micropropagation), genetic transformation and *In vitro* conservation (Machado *et al*., 2011). Tissue culture is the only methodology that can produce a large quantity of clonal plants in a short time with high phytosanitary quality. The development of new cheaper methods for *In vitro* culture is necessary. Some progress has been made, including automated methods of *In vitro* culture using bioreactors, which reduce costs due to the use of a liquid culture medium that eliminates the need for the more expensive agar. Moreover, the culture, using natural light and macro and micronutrients as precursors for plant growth regulators, has contributed to decreased costs. Alternative methods to produce plant growth regulators (Lopes da Silva *et al*., 2013) and chemical sterilization methods for culture media (Brondani *et al*., 2013) had been performed.

The use of nutrients from vinasse to develop a culture medium for the *In vitro* propagation of plants can decrease the environmental impact caused by this residue and, moreover, it decreases plant production costs. Currently, plant production using *In vitro* propagation is becoming more common. Biofactories are in constant expansion in many industrialized countries. In this context, we developed a plant culture medium using vinasse. The aim of this research was to demonstrate the efficiency of vinasse when used as the base for a culture medium as an alternative to vinasse disposal. This study is part of a patented process (Soccol *et al*., 2008) that was developed in our laboratory and is the first report on the use of vinasse in plant tissue culture.

Material and Methods

Vinasse source and preparation: Vinasse was collected in artificial ponds made of impermeable material. The vinasse stored in these ponds originated from the distillation of alcohol that was obtained from sugarcane juice. The vinasse was decanted and filtered, according to Santos *et al*., (2013). After the pretreatment of vinasse, the biochemical oxygen demand (BOD) was determined

by a five-day incubation, and the chemical oxygen demand (COD) was assessed by the open reflux method, both following the methodologies proposed by the APHA (Anon., 2005).

Vinasse culture media: To develop a vinasse culture medium, treated vinasse (decanted and filtered) was analyzed for the sulfate, chloride, phosphate, nitrate, ammonium, calcium, magnesium, sodium, potassium, manganese, zinc and total iron content. The sulfate content was quantified by the turbidimetric method, the chloride content was quantified by the titulometric method with mercury nitrate, the phosphate content was quantified by the colorimetric method (ascorbic acid) and the nitrogen content, as nitrate and ammonium, was quantified by the reduction method with cadmium and the phenate method, respectively. The calcium and magnesium contents were obtained by titulometry with EDTA (ethylenediamine tetraacetic acid), and the sodium and potassium contents were quantified by flame photometry (Anon., 2005). The total iron content was quantified by the phenanthroline method (Saywell and Cunningham, 1937). The micronutrients, such as zinc and manganese, were quantified by atomic absorption spectroscopy (Anon., 2005). We developed our vinasse medium based on the quantity of ions contained in the KC medium (Knudson, 1946) (Table 1).

Buffer effect in vinasse: The vinasse buffer effect was evaluated by using it as a culture medium. Treated vinasse was diluted at 5, 10 and 15% and was supplemented with 30 g L^{-1} sucrose and 0.3 mg L^{-1} BAP (6-Benzylaminopurine). The pH was measured and reported every seven days for 28 days.

Culture establishment: *Oncidium leucochilum* Batem. Ex Lindl. was used to test the vinasse culture media. Closed capsules of *O. leuchochilum* were disinfected by immersion in 70% ethanol for 1 min followed by immersion in NaOCl (2.5%) for 20 min. The capsules were then washed three times with distilled and autoclaved water and were then opened inside a laminar flow chamber, where the seeds were inoculated on half-strength MS salts (Murashige and Skoog, 1962) supplemented with 30 g L^{-1} sucrose and solidified with 6 g L^{-1} agar (Vetec®). The seedlings were *In vitro* propagated using the same medium supplemented with 0.3 mg L^{-1} BAP (Scheidt *et al*., 2009).

***In vitro* culture on vinasse dilutions:** To evaluate the effects of the dilution of vinasse, shoots (1 cm aerial part height) were cultured in liquid vinasse media at a 0, 2.5, 5 and 10% dilution. MS medium was used as a control to compare the vinasse dilutions. All media were supplemented with 30 g L^{-1} sucrose and 0.3 mg L^{-1} BAP. The leaf number, lateral shoot number, root number, height of the aerial part (cm), fresh mass (g), protuberance number, shoot percentage, rooting percentage, percentage of explants with some necrosis point and survival percentage were evaluated after 60 days of *In vitro* culture.

***In vitro* culture on MS medium with substitution of iron and calcium by vinasse ions:** To substitute the iron and calcium of the MS medium with the vinasse ions, $CaCl_2 \cdot 2H_2O$, $Na_2EDTA \cdot 2H_2O$ and $FeSO_4 \cdot 7H_2O$ were removed from the MS medium, which was then supplemented with 0, 2.5, 5 and 10% treated vinasse. Complete MS medium was used as control. All media were liquid and were supplemented with 30 g L^{-1} sucrose and 0.3 mg L^{-1} BAP. Shoots with 1.5 cm height were used as explants. All variables described above were analyzed after 60 days of *In vitro* culture, with exception of the protuberance number substituted with the hyperhydricity percentage.

Table 1. Ions contained in the KC medium (Knudson, 1946), in the KC vinasse media and in the 2.5% treated vinasse. KCV1 = 2.5% vinasse, 1000 mg L^{-1} $Ca(NO_3)_2 \cdot 4H_2O$ and 6.5 mg L^{-1} $MnSO_4 \cdot 4H_2O$, KCV2 = 2.5% vinasse and 1000 mg L^{-1} $Ca(NO_3)_2 \cdot 4H_2O$, KCV3 = 2.5% vinasse, 1000 mg L^{-1} $Ca(NO_3)_2 \cdot 4H_2O$, 6.5 mg L^{-1} $MnSO_4 \cdot 4H_2O$ and 170.6 mg L^{-1} $Ca_3(PO_4)_2$, KCV4 = 2.5% vinasse, 1000 mg L^{-1} $Ca(NO_3)_2 \cdot 4H_2O$, 6.5 mg L^{-1} $MnSO_4 \cdot 4H_2O$ and 120 mg L^{-1} $NaH_2PO_4 \cdot H_2O$, KCV5 = 2.5% vinasse, 1000 mg L^{-1} $Ca(NO_3)_2 \cdot 4H_2O$, 6.5 mg L^{-1} $MnSO_4 \cdot 4H_2O$ and 240 mg L^{-1} $NaH_2PO_4 \cdot H_2O$. All vinasse used for media formulation was pretreated (decanted and filtered).

Ion (mg.L^{-1})	KC	KCV1	KCV2	KCV3	KCV4	KCV5	2.5% treated vinasse
Ca	169.72	210.77	210.77	276.90	210.77	210.77	41.05
NO_3	525.12	525.14	525.14	525.14	525.14	525.14	0.0165
NH_4	136.51	1.1912	1.1912	1.1912	1.1912	1.1912	1.1912
SO_4	472.76	39.25	36.45	39.25	39.25	39.25	36.45
Mg	24.65	2.545	2.545	2.545	2.545	2.545	2.545
Fe	5.02	0.698	0.698	0.698	0.698	0.698	0.698
Mn	1.85	1.67	0.072	1.67	1.67	1.67	0.072
K	71.83	44	44	44	44	44	44
PO_4	174.47	9.5	9.5	113.97	92.08	174.68	9.5
Na	-	0.175	0.175	0.175	20.17	40.16	0.175
Cl	-	0.927	0.927	0.927	0.927	0.927	0.927
Zn	-	0.019	0.019	0.019	0.019	0.019	0.019

***In vitro* multiplication on different KC vinasse media:** To induce multiple shoots in explants, different KC vinasse media were tested. The media were: KC (control), KCV1, KCV2, KCV3, KCV4 and KCV5. All media were supplemented with 30 g L^{-1} sucrose and 0.3 mg L^{-1} BAP and were solidified with 6 g L^{-1} agar. KC medium was used as a control. All variables described above were analyzed after 60 days of *In vitro* culture.

***In vitro* elongation and rooting on different KC vinasse media:** Shoots (1 cm aerial part height) were cultured in three media: KC (control), KCV1 and KCV5. All media were supplemented with 30 g L^{-1} sucrose and 1 g L^{-1} activated charcoal and were solidified with 6 g L^{-1} agar. The shoot number, root number, height of the aerial part (cm), fresh mass (g), shoot percentage, root percentage, protuberance percentage and survival percentage were evaluated after seven months of *In vitro* culture.

Acclimatization: Plantlets from the elongation and rooting media were removed from the flasks, and their roots were washed with tap water (faucet). Complete plantlets were obtained in KCV1 and KCV5 only; therefore, only plantlets originating from these two media were acclimatized. The plantlets were cultured in substrate coconut fiber in the greenhouse with intermittent nebulization for 14 days. After two weeks of intermittent nebulization, the plantlets were transferred to a greenhouse with manual irrigation. Leaf fertilizer was applied every week. This fertilizer consisted of NPK (06-06-08) + micronutrients, Ca, S, Mg, B, Cu, Zn (01-0.5-0.5-0.03-0.1-0.1). The survival percentage was evaluated after 28 days of *ex vitro* culture.

Culture conditions and statistical analysis: All media had their pH adjusted to 5.7 and were autoclaved at 1 atm and 121ºC for 20 min. The cultures were kept at 25 ± 2ºC under white fluorescent light (28 μM m^{-2} s^{-1}) with a 16 h photoperiod. The experimental design was completely randomized with five replicates of ten explants. The data were submitted to a normality analysis using the Lilliefor's test followed by an analysis of variance (ANOVA) and Duncan´s test, both at a $p<0.05$. The counting variables were transformed to $\sqrt{x+0.5}$, and the percentage variables were transformed to $\arcsin\sqrt{x/100}$. All statistical analyses were performed with the GENES software (Cruz, 2001).

Results and discussion

Nutrient determination, biochemical oxygen demand (BOD) and chemical oxygen demand (COD): All nutrients present in KC medium were found in the treated vinasse (Table 1). Moreover, several nutrients such as Na, Cl and Zn that were not present in KC medium were found in treated vinasse (Table 1). The treated vinasse had a greater quantity of nutrients than the KC medium (data not shown); therefore, it must be diluted prior to use. A 10% vinasse dilution was used for the establishment of a hydroponics solution, which produced results similar to a commercial solution (Santos *et al*., 2013). This result suggests that a high vinasse dilution was suitable for plant growth. The nutrients found in a greater amount in the vinasse dilution were Ca, K and SO_4 (Table 1). Similar results were found in vinasse from sugarcane, where a high content of calcium and potassium was observed (Almeida, 1952; Glória, 1976).

The BOD is the oxygen amount consumed during the degradation of organic matter by a biological process. This measure is associated with the toxicity of the waste to aquatic life; high levels of BOD cause suffocation of aquatic fauna and flora resulting in the death of aquatic life. The BOD limit level for effluents before they are dispersed into the environment is 50 mg L^{-1} (Anon., 1989). The BOD of pure vinasse is between 20,000 and 35,000 mg L^{-1}, meaning that this waste is extremely harmful to the fauna of lakes and rivers (Da Silva *et al*., 2007); however, the treated vinasse (decanted and filtered) used in the present study has a lower, but still high, BOD of 6,524.6 mg L^{-1}.

The COD is the amount of oxygen consumed using an oxidizing agent under controlled conditions; nevertheless, environmentally, COD represents an indirect measure that indicates the amount of organic compounds in water. Normally the COD is higher than the BOD because the oxidizing agents are more aggressive under the controlled conditions. The acceptable COD level of an effluent to be dispersed in the environment is 1000 mg L^{-1} (Anon., 1989). Pure vinasse is between 50,000 and 150,000 mg L^{-1} COD (Pant & Adholeya, 2007). The treated vinasse had a COD of 7808.6 mg L^{-1}, which was still far over the limit. The vinasse will be well diluted prior to use, below 10%, which will further reduce the COD value.

Buffer effect in vinasse: After seven days, the vinasse pH decreased from 5.7 to approximately 5.4; at days 14 and 21 the pH increased, and at 28 days the pH was almost stable in the 5 and 10% vinasse, whereas the pH in the 15% vinasse had decreased (Fig. 1). Different vinasse dilutions had similar behaviors, but smaller vinasse dilutions showed a reduced trend toward acidification (Fig. 1). These results are similar to those observed during the application of the pure vinasse in the field; initially the soil is acidified, and after some time the pH returns to near the initial value (Almeida, 1952).

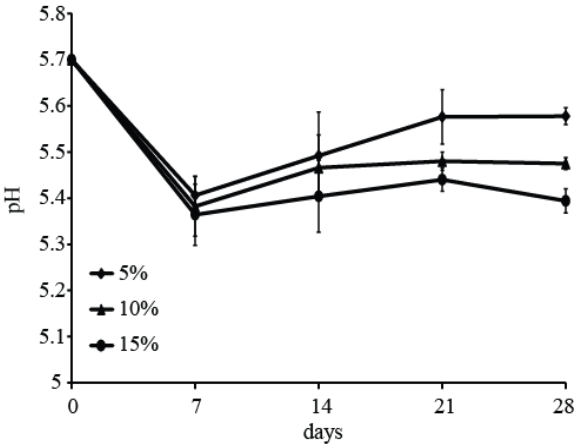

Fig. 1. Buffer effect in liquid media composed of different vinasse dilutions (5, 10 and 15%). The pH was measured each week until 28 days. Error bars correspond to the standard deviation of the mean.

The appropriate range of pH for the *In vitro* germination of orchids is from 4.5 to 5.5 (Knudson, 1946). However, for the *In vitro* culture of different orchid explants, a pH of up to 5.8 has been used (Knapp *et al*., 2000; Chapla *et al*., 2009). Hence, the buffer effect of the culture medium must be stable, allowing little variation. Different vinasse dilutions presented relative buffer effects, which did not vary outside of the range appropriate for orchid explant culture.

Culture on vinasse dilutions: The survival percentage of explants were not significantly different among the MS (control) and 0 and 2.5% vinasse, varying from 95.8 to 100%; however, the 5 and 10% vinasse dilutions were significantly different from the control, varying from 83.3 and 50% (Table 2). The vinasse dilution is an important variable in formulating culture medium due its direct influence on the explant survival. Smaller vinasse dilutions (5 and 10%) possessed a significant phytotoxic effect on explants.

The absence of vinasse decreased the percentage of leaves with several necroses, suggesting that this orchid is sensitive to the presence of inorganic salts that were added to the culture medium (Table 2). The MS medium used as a control showed the best results for shoot number, protuberance number and fresh mass (Table 2); nevertheless, the medium with 2.5% vinasse presented results for shoot height and shoot percentage similar to those found with the MS medium. Moreover, the medium with 2.5% vinasse showed superior results for leaf number, root number and rooting percentage (Table 2). The 2.5% vinasse dilution was ideal for plant tissue culture and did not affect explant survival. This dilution also provided the best results for some of the growth parameters.

***In vitro* culture on the MS medium with the substitution of iron and calcium with vinasse ions:** In this experiment, there were no significant differences for leaf number and root percentage (Table 3). The MS medium (control) had a greater shoot number, but the MS (control) and the 2.5% and 5% vinasse did not show a significant difference for root number, shoot height or fresh mass. For *In vitro* multiplication, the shoot number (propagules) was inferior to the control, which is not useful for shoot proliferation. Even so, this medium could be used to promote the elongation and rooting of the explants because the same results for the number or roots, shoot height and fresh mass were obtained for the control and the 2.5 and 5% vinasse.

Shoot and survival percentage did not show a significant difference for the MS (control) and the MS supplemented with 0, 2.5 and 5% vinasse but was significantly greater than the 10%vinasse (Table 3). Hyperhydricity was observed in the absence of iron and calcium (0% vinasse) in approximately 60% of the explants. Liquid culture is the most common cause of hyperhydricity in plant tissue culture (Scheidt *et al*., 2011). Some species are more resistant than others (Carvalho *et al*., 2013); nevertheless, *O. leucochilum* can be cultivated *In vitro* in liquid medium without the occurrence of hyperhydricity. This result was most likely caused by an iron and calcium deficiency; in studies on calcium deficiency in tissue cultures, it was shown that an increase in calcium in the medium reduced hyperhydricity and tip necrosis (Sha *et al*., 1985; Machado *et al*., 2014). Moreover, increases in calcium have been effective in eliminating hyperhydricity in quince (*Cydonia oblonga*) cultures (Singha *et al*., 1990). Increasing the concentration of iron in a medium with 0.7–0.8% agar during micropropagation of carnation (*Dianthus caryophyllus*) is reported to reduce hyperhydricity in the cultures (Yadav *et al*., 2003).

Table 2. *In vitro* multiplication of *Oncicium leucochilum* in different vinasse dilutions. Leaf number (LN), shoot number (NB), root number (RN), shoot height (SH cm), fresh mass (FM g), protuberance number (PN), shoot percentage (SP %), rooting percentage (R %), percentage of explants with some necrosed region (N %) and survival percentage (S %) are shown. All media were liquid, not shaken and supplemented with 30 g L^{-1} sucrose and 0.3 mg L^{-1} BAP (6-benzylaminopurine).

Treatment	LN	NB	RN	SH cm	FM mg
MS (control)	7.46 b	1.37 a	1.00 ab	2.66 a	320.5 a
0% vinasse	4.75 c	0.15 c	0.23 c	1.97 b	127.7 b
2.5% vinasse	10.06 a	0.54 b	1.27 a	2.45 a	150.2 b
5% vinasse	5.82 c	0.33 b	0.40 bc	1.93 b	109.6 b
10% vinasse	5.44 c	0.40 b	0.28 c	1.95 b	109.8 b
CV(%)	5.8	5.6	17.0	7.9	17.1
Treatment	**PN**	**SP %**	**R %**	**N %**	**S %**
MS (control)	16.6 a	45.8 a	27.1 ab	91.6 b	100 a
0% vinasse	0.6 b	20.8 b	18.7 b	66.6 a	95.8 a
2.5% vinasse	0.2 b	41.6 a	45.8 a	100 c	100 a
5% vinasse	0.3 b	27.5 b	26.6 ab	100 c	83.3 b
10% vinasse	0.0 b	20.8 b	12.5 b	100 c	52.1 c
CV(%)	22.7	9.3	26.8	7.4	7.4

[1] Means within a column followed by the same letter for each parameter are not different at $p<0.05$ by Duncan's test

Table 3. *In vitro* multiplication of *Oncicium leucochilum* in different vinasse dilutions supplemented with MS medium (Murashige and Skoog, 1962) without $CaCl_2 \cdot 2H_2O$, $Na_2EDTA \cdot 2H_2O$ and $FeSO_4 \cdot 7H_2O$ after 60 days of culture. The MS medium (control) was used complete. Leaf number (LN), shoot number (SN), root number (RN), shoot height (SH cm), fresh mass (FM g), protuberance number (PN), shoot percentage (SP %), root percentage (R %), hyperhydricity percentage (H %) and survival percentage (S %) are shown.

Treatment	LN	SN	RN	SH cm	FM g
MS (control)	12.1 a[1]	3.8 a	7.1 ab	3.4 ab	1.038 a
0% vinasse	10.3 a	2.0 b	3.2 c	2.4 c	0.628 bc
2.5% vinasse	11.1 a	2.0 b	7.6 a	3.8 a	0.847 ab
5% vinasse	15.5 a	2.1 b	8.2 a	3.8 a	0.877 ab
10% vinasse	12.7 a	1.4 b	4.1 bc	2.8 bc	0.413 c
CV(%)	12.0	17.5	21.4	17.1	12.3
Treatment	**PN**	**SP %**	**R %**	**H %**	**S %**
MS (control)	21.2 a	75.0 a	87.5 a	0.0 b	100 a
0% vinasse	19.9 a	86.6 a	58.3 a	59.2 a	100 a
2.5% vinasse	16.3 a	83.0 a	90.0 a	0.0 b	100 a
5% vinasse	3.6 b	91.6 a	90.0 a	0.0 b	100 a
10% vinasse	1.3 b	35.0 b	77.0 a	0.0 b	60 b
CV(%)	31.9	24.1	27.4	23.2	12.3

[1] Means within a column followed by the same letter for each parameter are not different at $p<0.05$ by Duncan's test

Protuberances were observed in all treatments and were highest in the MS (control) and the 0 and 2.5% vinasse (Table 3). The vinasse might possess a substance that inhibits the formation of protuberances because these structures are produced in larger number in the largest vinasse dilutions and decrease in number in the lowest vinasse dilutions. Moreover, the inhibitory effect of the vinasse on protuberance induction might be associated with the presence of ethanol residues in vinasse because ethanol is an inhibitor of somatic embryogenesis (Caldas *et al*., 1998), and the protuberances of orchids are similar to somatic embryos, as suggested by the name protocorm-like bodies.

The possibility of an *In vitro* culture in liquid media is advantageous due to the production cost reduction by removing the need for agar (i.e., the most expensive component of culture media) and the process automation by using bioreactors. Other advantages of this experiment are that it was conducted in stationary culture, which also reduced production costs because the electricity that is required to move shakers is not necessary (Lopes da Silva *et al*., 2012).

***In vitro* multiplication on different KC vinasse media:** There were no significant differences for shoot number, root number, rooting percentage and survival percentage (Table 4). The KC medium (control) was superior for protuberance number when compared to other KC formulations. KCV2 and KCV3 presented higher rates of leaves with necrosis, 87.5 and 62.5%, respectively (Table 4). KCV2 had a lower amount of manganese (0.072 mg L^{-1} Mn) compared to the other formulations, KC (1.85 mg L^{-1} Mn) and KCV1, KCV3, KCV4 and KCV5 (1.67 mg L^{-1} Mn). The main symptom of manganese deficiency is internerval chlorosis associated with small necrosed points (Taiz & Zeiger, 2004). The higher rate of leaves with necrosis in the KCV3 formulation was not caused by a manganese deficiency, nevertheless it is possible that it could be provoked by the use of $Ca_3(PO_4)_2$ as the source of phosphate. Similar results were found in the protuberances of *Encyclia boothiana* (orchid), where $Ca_3(PO_4)_2$ promoted phytotoxicity leading to death after 120 days of *In vitro* culture (Stenberg & Kane, 1998). The lowest rate of shoot percentage was found in KCV2. This may also be attributed to the low amount of manganese in this formulation because manganese is responsible for enzyme activation of the tricarboxylic acid cycle, which is associated with energy production in plant cells (Taiz & Zeiger, 2004).

The best result for shoot height was found in KC (control); nevertheless, it was not significantly different from KCV1, KCV2, KCV4 and KCV5 (Table 4). The reduced shoot height observed in the explants of KCV3 was caused by the use of $Ca_3(PO_4)_2$, which likely influenced the higher rate of leaves with necrosis, as discussed above. The best results for leaf number were found in KC (control), KCV4 and KCV5, most likely due to a higher amount of phosphate present in these formulations, 174.47, 92.08 and 174.68 mg L^{-1} PO_4, respectively. Although KCV3 has 113.97 mg L^{-1} PO_4, this source of phosphate has low solubility and the precipitated phosphate is unavailable to the explants. This fact can also be used to explain why the explants cultivated in the KCV3 medium presented low fresh mass compared to KC and KCV5 (Table 4). The best results for fresh mass were obtained in KC (control) and KCV5, which were significantly different than the other KC formulations. The KC and KCV5 possessed the same level of phosphate, which suggested the influence of this ion in increasing the fresh mass. This ion is needed by plants in large quantities. In this study, the levels of phosphate used in all of the KC formulations were not harmful to the development of the explants. Some KC formulations just decreased some of the parameters of growth.

Table 4. *In vitro* multiplication of *Oncicium leucochilum* on KC medium (Knudson 1946) and on two KC formulations composed of 2.5% treated vinasse (decanted and filtered), (KCV1 = 1000 mg L^{-1} $Ca(NO_3)_2 \cdot 4H_2O$ and 65 mg L^{-1} $MnSO_4 \cdot 4H_2O$), (KCV2 = 1000 mg L^{-1} $Ca(NO_3)_2 \cdot 4H_2O$), (KCV3 = 1000 mg L^{-1} $Ca(NO_3)_2 \cdot 4H_2O$, 65 mg L^{-1} $MnSO_4 \cdot 4H_2O$ and 170.6 mg L^{-1} $Ca_3(PO_4)_2$), (KCV4 = 1000 mg L^{-1} $Ca(NO_3)_2 \cdot 4H_2O$, 65 mg L^{-1} $MnSO_4 \cdot 4H_2O$ and 120 mg L^{-1} $NaH_2PO_4 \cdot H_2O$) and (KCV5 = 1000 mg L^{-1} $Ca(NO_3)_2 \cdot 4H_2O$, 65 mg L^{-1} $MnSO_4 \cdot 4H_2O$ and 240 mg L^{-1} $NaH_2PO_4 \cdot H_2O$) after 60 days of culture. Leaf number (LN), shoot number (SN), root number (RN), shoot height (SH cm), fresh mass (FM g), protuberance number (PN), shoot percentage (SP %), rooting percentage (R %), percentage of explants with some necrosed region (N %) and survival percentage (S %) are shown. All media were supplemented with 30 g L^{-1} sucrose and 0.3 mg L^{-1} BAP (6-benzylaminopurine) and were solidified with 6 g L^{-1} agar.

Treatment	LN	SN	RN	SH cm	FM g
KC (control)[2]	16.0 ab[1]	3.7 a	0.6 a	2.8 a	0.266 a
KCV1 vinasse	13.1 bc	3.3 a	0.7 a	2.7 ab	0.129 b
KCV2 vinasse	12.4 c	2.4 a	0.7 a	2.5 abc	0.131 b
KCV3 vinasse	13.0 bc	2.3 a	0.5 a	2.4 bc	0.124 b
KCV4 vinasse	17.5 a	4.4 a	0.5 a	2.5 abc	0.102 b
KCV5 vinasse	15.1 abc	3.6 a	0.5 a	2.6 ab	0.224 a
CV(%)	5.6	12.3	19.4	6.4	17.3
Treatment	**PN**	**N %**	**R %**	**SP %**	**S %**
KC (control)	18.6 a	0 d	37.5 a	100 a	100 a
KCV1 vinasse	0 b	37.5 bc	50 a	100 a	100 a
KCV2 vinasse	0 b	87.5 a	50 a	87.5 b	100 a
KCV3 vinasse	0 b	62.5 ab	50 a	100 a	100 a
KCV4 vinasse	1.5 b	12.5 dc	25 a	100 a	100 a
KCV5 vinasse	0.4 b	37.5 bc	50 a	100 a	100 a
CV(%)	22.9	44.7	53.7	7.2	0

[1] Means within a column followed by the same letter for each parameter are not different at $p<0.05$ by Duncan's test

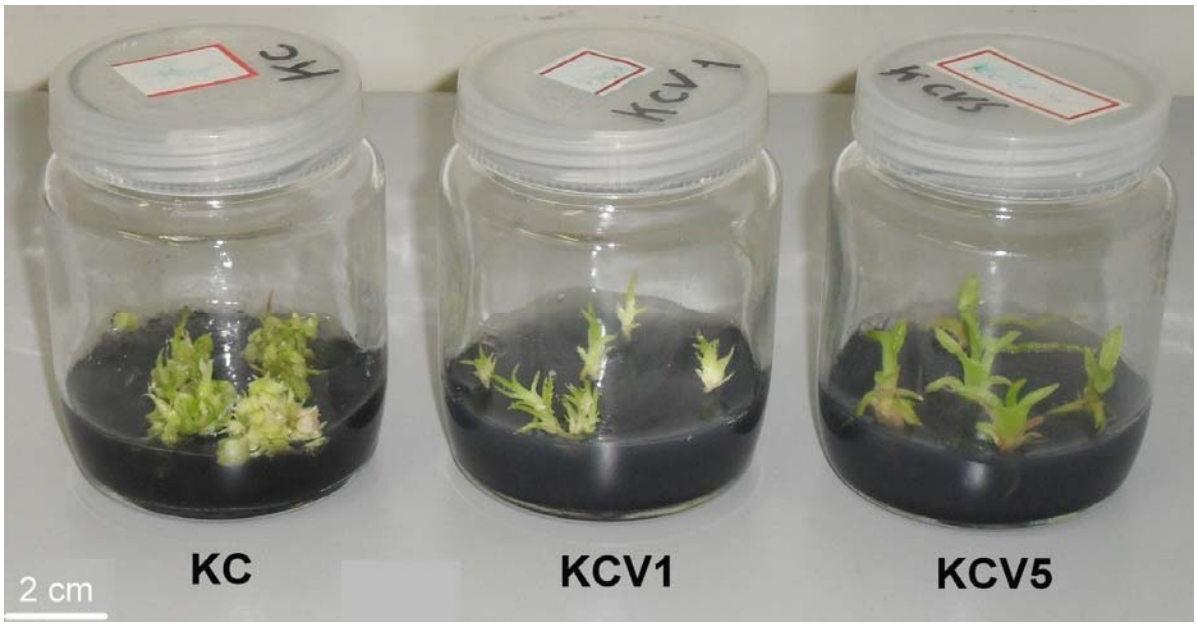

Fig. 2. *In vitro* elongation and rooting of *Oncidium leucochilum* on KC medium (control) and two KC formulations with 2.5% vinasse after six months. Media are free of plant growth regulators.

***In vitro* elongation and rooting on different KC vinasse media:** The vinasse media were superior to the KC medium in promoting *In vitro* elongation and rooting (Fig. 2). The KC medium promoted a low rooting rate (8%) compared to the 68 and 100% obtained by KCV1 and KCV5, respectively (Table 5). Moreover, plantlets cultured on the KC medium only became protocorm-like body clusters (Fig. 2), making it impossible to acclimatize these explants. The result for root number was also higher in vinasse media than KC (control), with KCV5 (3.8 roots per explant) being the greatest followed by KCV1 (1.8 roots per explant). The KC (control) presented the lowest

root number (0.1 root per explant). The KC promoted the highest number of protocorm-like bodies, which impeded root formation and elongation in plants (Fig. 2). The vinasse media presented inhibition toward the formation of protocorm-like bodies. The KCV1 and KCV5 did not show statistically different values for shoot height (Table 5). It is common for the explants that were removed from the multiplication medium to continue to proliferate due to the endogenous presence of the cytokinins used to induce the *In vitro* multiplication of buds, protuberances and shoots, as demonstrated in several reports. For example, in *Dyckia maritima,* it was suggested that prior to a phase of rooting, submitting the clusters of shoots to a shoot elongation phase would avoid new shoot formation (Lopes da Silva *et al*., 2004). When explants cultivated on *In vitro* rooting media are difficult to root, an *In vitro* elongation medium can be used prior to transfer to the *In vitro* rooting medium. The phase of *In vitro* elongation and rooting can be conducted simultaneously (with one subculture, one medium and by using isolated shoots) or separately (with two subcultures and two different or the same media, using the first to subculture shoot or bud clusters and the second to subculture isolated shoots). The prior elongation of shoot clusters is necessary when explants continue to multiply and the rooting is not suitable. However, in *Vriesea fosteriana,* the supplementation of 0.54 μM NAA (1-naphthaleneacetic acid) was necessary to stop these lateral shoot proliferations as well as to return apical growth of the shoots. In this way, rooting was easily induced (Mercier and Kerbauy, 1992). However, the vinasse appears to possess a compound that can avoid protocorm-like body proliferation, which would be useful for plant tissue culture and must be further investigated. The vinasse media decreased the shoot percentage but did not affect the shoot number, which was not significantly different than in the KC medium. This result is suitable for the induction of *In vitro* rooting as discussed above. The survival percentage was 100% in all treatments (Table 5).

Acclimatization: At least one elongated shoot is necessary for the acclimatization of micropropagated plants. The KC medium did not produce suitable elongated shoots, which impeded the attempt to acclimatize these explants (Table 5 and Fig. 2). Different culture media can influence plant survival during acclimatization, as demonstrated in microshoots of *Melaleuca alternifolia* that were cultivated in half-strength MS and full-strength MS, resulting in 80 and 100% survival, respectively (Oliveira *et al*., 2010). Plantlets elongated and rooted on KCV1 and KCV5 media were successfully acclimatized with a 91% survival rate for both KC vinasse formulations. This result indicated that vinasse could be used successfully to develop plant tissue culture. A complete protocol for the micropropagation of *O. leucochilum* was established with high rates of plant survival. Acclimatization protocols with survival rates above 90% are suitable for all plant species. In this phase, a great number of plants did not survive due to hard stress that was caused by the transfer from *In vitro* conditions to *ex vitro* conditions (Schuck *et al*., 2012). Low survival rates limit the commercial use of micropropagation. This protocol for micropropagation, using vinasse media, can be adapted for other plant species, as observed in our laboratory with bromeliads, which were also successfully micropropagated.

Table 5. *In vitro* elongation and rooting of *Oncidium leucochilum* cultured on vinasse media after eight months. Rooting (R %), root number (RN), height of the aerial part (HPA cm), survival (S %), fresh mass (FM g), protuberances (P %), shoot (Sh %) and shoot number (SN) are shown.

Treatment	R %	RN	HPA cm	S %
KC (control)	8 c[1]	0.1 c	0.87 b	100 a
KCV1	68 b	1.8 b	1.58 a	100 a
KCV5	100 a	3.8 a	1.99 a	100 a
CV(%)	17.8	12.6	21.3	0
Treatment	**FM g**	**P %**	**Sh %**	**SN**
KC (control)	0.399 a	100 a	100 a	2.2 a
KCV1	0.114 b	8 b	72 b	2.6 a
KCV5	0.171 b	0 b	64 b	2.6 a
CV(%)	56.9	31.0	29.8	15.3

[1] Means within a column followed by the same letter for each parameter are not different at $p<0.05$ by Duncan's test

Conclusions

It was possible to develop a plant culture medium using vinasse that resulted in a suitable *In vitro* culture medium for *Oncidium leucochilum* (orchid). The best vinasse dilution (decanted and filtered) was 2.5%. The vinasse dilutions demonstrated a good buffer effect. The best KC formulations with vinasse were KCV1 and KCV5, and the KCV5 was superior to KCV1 for *In vitro* rooting. For *In vitro* multiplication, the results were similar among the vinasse formulations (KCV1 and KCV5) and the KC medium (control), with the exception of protocorm-like body number, which was smaller in the vinasse formulations. For *In vitro* elongation and rooting, the vinasse formulations were superior to the KC medium. The micropropagated plantlets that were obtained were successfully acclimatized, demonstrating the efficacy of the use of vinasse to formulate plant culture media. This shows the great potential of this technology as a rational alternative to vinasse disposal.

Acknowledgements

The authors thank CNPq (the National Council for Scientific and Technological Development) and Capes (Coordination for the Improvement of Higher Level - or - Education-Personnel) for the Ph.D. and M.Sc scholarships. Moreover, we thank the Fundação Araucária for supporting this research.

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(Received for publication 20 June 2013)