The Influence Of Surface Sterilization Treatments On The Initiation Of Callus Cultures In *Vanilla planifolia*

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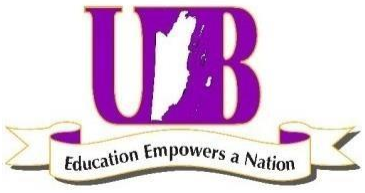
Submitted by

Faith Madubuko

Under the Supervision of

Dr. Dion Daniels



Department of Science

Faculty of Science and Technology

University of Belize

Abstract

Vanilla planifolia (Order: Orchidales, Family: Orchidacea), is an herbaceous climbing orchid and a tropical commercial spice crop from which the popular flavouring substance, vanillin is produced. The demand for vanillin is so high and the cost so exorbitant that over 97% of vanillin used as a flavour and fragrance is synthetic. The country of Belize has a favourable environment for vanilla production and the crop possesses huge potential to be tapped into as it relates to local sale and international export. Vanilla can also form an important part in the diversification of the nation's agrarian sector. The interest of investors can be harnessed for the production of vanilla so long as planting material supply is reliable and sufficient. This study examines different ways that vanilla explant tissues can be efficiently propagated in vitro. Four different treatments were used to gauge the influence of different explant surface sterilization methods; three different concentrations of sodium hypochlorite (3%, 4%, 5%, respectively) and 70% ethanol were used. After surface sterilization, the V. planifolia explants were inoculated onto the same medium; 10 mL/0.5 L of Murashige-Skoog (MS) salts, 10 mL/0.5 L MS vitamins, 5 mL/0.5 L of nicotinic acid, 1 ml/0.5 L of 6-Benzylaminopurine (6-BAP) and 4 g/0.5 L of Agar. Accessibility to an established protocol of the kind established in this study will be a key step towards the establishment of large scale production of vanilla in Belize.

Acknowledgements

Throughout the process of performing the activities outlined in this paper and compiling the results and analyses in the manner stated therein was not easy, there have been people who have contributed enormously to the final product. To begin with, I would like to show my gratitude to Dr. Dion Daniels, lecturer at the University of Belize and my program coordinator and supervisor for providing insight and information, both of which were indispensable to the organization of this report. He was willing to answer any and all queries that I had and coupled those answers with guidance and technical know-how on how best to produce the subjects so meticulously delineated in this paper.

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Introduction

Vanilla is among the most popular flavors in the world and was first used by the Aztec people in Mexico as a flavour aid to cocoa. As a result of the high vanillin content, it has been named one of the premier sources of vanilla flavouring. The seeds of the pod are used to flavour ice cream, soft drinks, candies and liquor (Zuraida, et al., 2013). Vanillin has also found use in the production of cosmetics, pharmaceuticals and tobacco as well as condiments and the perfumery industry. *V. planifolia* grows wild in tropical forests and as a member of the Vanilla genus, it is the only one to produce an agricultural crop that is valuable. With the world production of *Vanilla* beans estimated at 3500 tonnes per annum, The Malagasy Republic grows 70 to 80% percent of the world's *Vanilla* crop (Kalimuthu, Senthilkumar, & Murugalatha, 2006). This amounts to nearly 7% of the total value of the world's spice trade.

Over here in this side of the world, Mexico has been the sole exporter of vanilla to the rest of the world due to the orchids in Mexico (particularly the ones in Veracruz) being pollinated by bees as opposed to being pollinated by hand as was accomplished in other sites around the world (Mexican Vanilla – A History, 2014). But over time, the need for the extract outweighed the cost of hand pollination that growing of the plant elsewhere required, to such extent that other countries such as China, Indonesia and Uganda have been able to overtake Mexico as the leading producers of vanilla. In addition, several reports now state that poor weather conditions are also a contributing factor when it comes to driving down the production of vanilla from Mexico and India. That is why countries such as Madagascar have been able to see an increase in their wholesale prices due to the decrease in production from Mexico. Looking at the list of the top five countries that produce vanilla beans, Mexico is the only country from Latin America or this side of the world for that matter to feature on that list; and it sits in fourth place.

If the economy in Belize is to undergo extensive development, its economy must be equally strong to support it. An avenue towards the strengthening the economy can be accomplished though the diversification of its agrarian interests. This can include looking at agricultural endeavours that virtually involve the same techniques and seeing if the same results can be achieved when applied to different products. An example of this can be the concept of micropropagation being applied to the cultivation of *V. planifolia*.

Research into the topic of micropropagation has unearthed the fact that there are laboratories in Belize that are active participants in the prospect of the micropropagation of plants such as banana, sugar and cassava. In fact, programs involving such plants have even progressed to such s stage that the labs are selling these plans that came about as a result of the employment of micropropagative techniques, to farmers and successes have been reported in the field (Daniels, 2014). The growth of this orchid and possible commercialization of the extract can become one more viable venture that the country can look towards as a source of cash flow. It can also help supplement gains from the citrus industry when profits from that industry are low or when losses are too high.

With all of that in mind, this research looks at the establishment of a protocol for explant surface sterilization that would reduce the risk of contamination and the waste of resources such as explant source material from the field.

Literature Review

Classification

Kingdom: Plantae

Division: Tracheophyta

Class: Magnoliopsida

Order: Asparagales

Family: Orchidaceae

Genus: *Vanilla*

Species: *V. Planifolia*

Vanilla is an herbaceous perennial climbing plant that utilises its roots for support. The vanilla bean (also known as the vanilla fruit), is one of the priciest spices in the market because its growth and harvest is a time-consuming and labour intensive project (Mujar, Sidik, Sulong, Jaapar, & Othman, 2014). The origin of the vanilla plant can be traced to the tropical area in Mexico and Central America and also the forest around South America (Moorthy and Moorthy, 2002 as cited in (Mushimiyimana, et al., 2011)).The flowers are a greenish-yellow, with a diameter of 2 inches. They only last a day and if they are to be pollinated, usually this is done by hand in the morning. The plants undergo self-fertilization, simply requiring the transfer of pollen from the anther to the stigma. The vanilla fruit is only produced on mature plants and they are generally over 10ft long. After being left to mature for five month, they are harvested and cured. The process of curing dries and ferments the pods while limiting the amount of essential oils that are lost. It is from this portion that the vanilla extract is obtained from the plant.

The propagation of vanilla traditionally is done by stem culture. But as was mentioned in the previous paragraph, growing the plant in this manner is very slow and tedious. Also, the numbers of seedlings that are produced by cuttings are few, rendering this method inefficient as it concerns meeting the national demand for quality planting materials. This is where the exploitation of the potentials of tissue culture for efficient multiplication and supply of required planting materials for large scale cultivation purposes in the country.

When it comes to the judging the degree of success of different surface sterilization methods particularly where it concerned the use of sodium hypochlorite and ethanol, a lot of information could not be gathered on that score. Also, the explants that were used in this case emanated from leaves. Prior research being done on the influence of surface sterilization methods focused on the use of nodes and seed parts (Zuraida, et al., 2013). Other treatments that have been involved in surface sterilization of *V. planifolia* include gamma irradiation and methyl jasmonate. In this particular report however, it was reported that low doses of both gamma irradiation and methyl jasmonate were sufficient to generate a positive result compared to using a high dose which gave a negative result (Mujar, Sidik, Sulong, Jaapar, & Othman, 2014).

Materials and Methods

This research was conducted at the University of Belize's Micropropagation Laboratory at Central Campus in Belmopan City under the Faculty of Science and Technology. It was carried out from April to May 2015

Plant Material

4 *V. planifolia* leaves, genus Vanilla were obtained from the green house located towards the back of the University of Belize Belmopan Campus, Belmopan City, Cayo District. They were cut from the mother plant and transported in plastic bags to the lab where the experiments were performed. They were then washed with distilled water to render them ready for the exercise.

Culture medium

10 mL/0.5 L Murashige-Skoog (1962) salts, 10 mL/0.5 L MS vitamins, 5 mL/0.5 L nicotinic acid and 1 ml/0.5 L 6-Benzylaminopurine (6-BAP) and 1 mg/L Agar. Sodium hydroxide and/or hydrochloric acid was used to adjust the pH of the solution to 5.8. A total of 17 jars were used to house the culture medium and the explants that were inoculated on them.

Instruments

Using a vertical autoclave, tiles, scalpels and forceps were sterilized in a vertical autoclave for 25 minutes at 121^{o}C. Scalpels were placed in 70% ethanol and flamed before and after use.

Experiment

The following sterilizing agents, ethanol 70%, sodium hypochlorite 3%, 4% and 5% were tested to determine which was the most effective as it concerned the establishment of *V. planifolia* leaf explants in vitro. The laminar flow chamber was turned on and all surfaces assiduously wiped down with 70% ethanol. It was then left on for 15 minutes before the explant sterilization exercise could begin. The 4 leaves obtained from the *V. planifolia* plant were then washed in a jar containing dishwashing solution for 15 minutes; this was accomplished by gently swirling the contents of the jar every 2 to 3 minutes. Afterwards, the leaves were then rinsed in distilled water for 5 minutes (a process also aided by gentle swirling). Each of the leaves were then placed, 1in each of the 4 jars in previously prepared solutions of 3% sodium hypochlorite, 4% sodium

hypochlorite, 5% sodium hypochlorite and the last one, 70% ethanol. It was ensured that the leaves were completely submerged in the solution. The leaf that was placed in the jar containing 70% ethanol was only left in there for 2 minutes and then it was taken out. After the appropriate amount of time had elapsed, all four leaves were taken out of their respective solutions and rinsed with distilled water for 5 minutes. Each leaf was cut into four strips of 5 cm^2. Each strip was further cut down to 4 explants of 0.5 cm^2. Each group of four explants were then inoculated onto jars containing the prepared medium. The jars were then place in a carton, closed and placed in the growth room at a temperature of 27+/-2 ^{o}c. Contaminants were evaluated 7 days after inoculation and the effects of the sterilizing agents were gauged.

Results and Discussion

The purpose of this experiment was to determine not just what surface sterilization agents worked best among the ones that were employed, but also at what concentrations those agents could be said to work best. The sterilization agents that were used were 3% sodium hypochlorite, 4% sodium hypochlorite, 5% sodium hypochlorite and 70% ethanol. As can be noted in Table 1, all 4 jars that contained explants that were sterilized with 4% sodium hypochlorite did not show any signs of contamination. They showed the best results compared to the other treatments. The 4 jars containing explants sterilized with 70% ethanol showed signs of fungal contamination. All explants were observed after a 7 day period.

	Surface Sterilization Treatments			
	3% NaOCl	**4% NaOCl**	**5% NaOCl**	**70% Ethanol**
Number of explants contaminated in each jar (4 explants in each jar)	**1**	**0**	**1**	**4**
	2	**0**	**1**	**4**
	2	**0**	**0***	**4**
	4	**0**	**0***	**4**
	3			

Table 1. Outcomes of surface sterilizing treatment *in vitro* of vanilla explants with sterilizing agents, 3% sodium hypochlorite, 4% sodium hypochlorite, 5% sodium hypochlorite and 70% ethanol; * - bacterial contamination

While the effort of contamination reduction was geared towards fungal contamination sources, an annotation was recorded as to the appearance of bacterial contamination as well.

Evidence of this can be seen in Table 2, where 8 explants in two jars that were sterilized with 5% sodium hypochlorite displayed signs of bacterial contamination.

5% NaOCl	
Number of explants showing fungal contamination	**Number of explants showing bacterial contamination**
1	**1**
1	**1**
0	**4**
0	**4**

Table 2. Comparison of instances of fungal contamination and bacterial contamination in the jar groups of different surface sterilization methods

Sodium hypochlorite is the main ingredient found in laundry bleach. Uses for it have been found in a plethora of areas including the paper and pulp industries, detergents, as a bleaching agent in the textile industry and as a compound for chlorinating the water in swimming pools. It can also be found in several cleaning products used in the kitchen and bathroom. In the food processing industry, sodium hypochlorite is employed in the sanitation of fruit and vegetable processing beef and poultry production, mushroom production and the sanitization of food preparation equipment.

This compound is a clear, slightly yellowish solution with a peculiar odor (Lenntech, 1998). When dissolved in water, it forms hypochlorous acid, H0Cl, a strong oxidising agent but a weak acid, and responsible for the characteristic bleaching effect produced. The hypochlorous acid produced has an antimicrobial activity as it relates to its ability to react with proteins and DNA of bacteria as well as breaking down their cell membranes. Usually, low concentrations of hypochlorous acid is required to achieve this effect. The sodium hypochlorite used in the experiment was obtained from Clorox bleaching fluid. While the original concentration was at

5.5%, the necessary dilutions were made to bring the sodium hypochlorite content down to the required markers of 3%, 4% and 5%.

The manner in which sodium hypochlorite is used and where such uses are employed in highly dependent on the concentration of sodium hypochlorite involved. At a concentration of 2%, sodium hypochlorite is utilised in the shock chlorination of wells. At a concentration of 3 to 6%, it is used a disinfectant in hospitals and in homes, in antisepsis of mammary glands in the dairy industry, maple syrup production, fish processing, sterilization of laboratory equipment and laundering clothes. Uses of sodium hypochlorite at 12 to 16% are left for the more heavy duty work for example, water and waste water treatment and swimming pool disinfectant. Sodium hypochlorite at high concentration is quite caustic and corrosive, resulting in burns and skin irritations were one to have dermal contact with it.

In concert with high concentrations, the decomposition rate of sodium increases as well. The transition metals such as nickel, copper and manganese as well as light and organic matter also accelerate the decomposition of sodium hypochlorite. Temperature is another factor that facilitates the decomposition of sodium hypochlorite. In summary, for every $10^{o}C$ increase in storage temperature, sodium hypochlorite decomposition could occur at a rate of approximately 3.5. Decomposition of liquid sodium hypochlorite leads to a decrease in strength and the formation of oxygen (Lister, 1956). In this case, more product will be needed if loss of sodium hypochlorite occurs, if it is to be used as a disinfectant or sterilization substance. First there is transformation into chlorate;

$3\ NaOCl \rightarrow 2\ NaCl + NaClO_3$

And then then the release of oxygen;

$2\ NaOCl \rightarrow 2\ NaCl + O_2$

This is particularly interesting to note given that in the experiment conducted, explants that were sterilized with sodium hypochlorite at 4% were relatively contamination free compared to those at 3% and 5%. However, multiple versions of this experiment will have to be performed for any conclusions reached to be established as being reasonable enough to build premises on, concerning the relationship between the strength of sodium hypochlorite and relative sterilization power.

Till today, the exact process behind the destruction of microorganisms by free chlorine has not been elucidated. The inactivation of these organisms by chlorine can be caused by a myriad of factors. From the loss of intracellular contents, breaks in DNA, decrease in the uptake of nutrients and the inhibition of protein synthesis to rind chlorination of amino acids and decreased adenosine triphosphate production (Centers for Disease Control and Prevention, 2008).

Ethanol (most commonly known as alcohol or ethyl alcohol) is the primary type of alcohol that is found in alcoholic beverages, birthed as a result of fermentation of sugars by yeasts. When consumed in sufficient amount, it is capable of inducing intoxication. There are varied uses for ethanol including use as a solvent, a fuel, antiseptic and sterilizing agent. The best way in which to describe the manner in which ethanol works in breaking down microbes is through the dehydration of cells, the disruption of membranes and the denaturation of proteins (Sridhar, 2008). Support of this mechanism emanates from the observation that ethyl alcohol, a dehydrating agent contains smaller amount of bacteria compared to mixtures of alcohol and water (Centers for Disease Control and Prevention, 2008).

70% ethanol was used as opposed to other concentrations of say 50% or even 100% due to the manner in which the alcohol/water mixture works. Haider (2012) goes on to state that at higher concentrations, the ethanol is less effective as the proteins of the bacterial cells are coagulated on impact. The alcohol is able to go through the cell wall of the organism in any direction but stops when it meets the ring of coagulated protein. This renders the cell inactive but not dead. When favourable conditions return, the cell is still ready to perform in all its infectious capacity. However, when 70% ethanol is poured on a single-celled organism, coagulation of the protein also occurs but at a slower rate. This helps it penetrate the cell all the way through before coagulation can kick in and stop its advancement. The entire cell is then subjected to coagulation and the organism dies. At lower concentrations, the ethanol is not as effective; it cannot break down all the lipids nor does it let water get into the cell.

The results obtained during the course of this experiment are by no means conclusive. Additional testing will have to be done multiple times to see if the results that were gained this first time remain the same. Other methods that were not previously employed as it relates to surface sterilization of *V. planifolia* will have to be examined as well.

Conclusions and Recommendations

The experiment demonstrates that the best surface sterilization treatment was 4% sodium hypochlorite (total time elapsed during leaf submersion in the treatment was 20 minutes). All explants were comparatively the same sizes so the degree of contamination found in each jar and around/on each explant could be attributed to the sterilisation treatment used. The presumed trend which was that the higher stronger the sterilization treatment used, the less contamination there will be was not followed; certain explants that were sterilised with 5% sodium hypochlorite were contaminated.

The cultivation of *V. planifolia* is an area that holds great promise as it relates to the diversification of Belize's agrarian economy. As such, it is recommended that more research is done on buttressing the veracity of the results achieved in this first experiment, and if there are deviations, taking note of them and attempt to piece out their sources. Experiments should be done not just on the concentrations of the two main sterilisation agents used here but also on others that apply to *V. planifolia* such as hydrogen peroxide, methyl jasmonate, Tween 20 and kocide solution. As there is no way currently to determine the level of contamination an explant showcases, the protocol and methods employed will have to be employed astringently to ensure that results obtained are as uniform as possible.

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