PROTOCOL DEVELOPMENT FOR MICRPROPAGATION OF VANILLA (*Vanilla planifolia* Andr.) CLONE (Van.2/05)

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By

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DEDICATION

This thesis is dedicated to my beloved family who paved my way towards education and for nursing me with affection and love and for their whole hearted partnership in the success of my life.

STATEMENT OF THE AUTHOR

First, I declare that this thesis is my work and that all sources of materials used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for M.Sc. degree at Jimma University and is reserved at the university library. I solemnly declare that this thesis is not submitted to any other institutions anywhere for award of any academic degree or certificate.

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BIOGRAPHICAL SKETCH

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Table of Contents

Contents **Page**

DEDICATION iii
STATEMENT OF THE AUTHOR iv
BIOGRAPHICAL SKETCH v
ACKNOWLEDGEMENTS vi
LIST OF TABLES ix
LIST OF FIGURES x
LIST OF APPENDEX TABLES xi
LIST OF ABBREVIATIONS xii
ABSTRACT xiii
1. INTRODUCTION 1
2. LITERATURE REVIEW 5
2.1. Origin and Distribution of Vanilla 5
2.2. Biology of Vanilla 5
2.3. Ecology and Agronomy of Vanilla 6
2.4. Production, Uses and Marketing of Vanilla 7
2.5. Constraints to Vanilla Production 9
2.6. Plant Tissue Culture and Its Application 11
2.7. Sequential Stages of Micropropagation 13
2.7.1. Stage 0: Preparation of stock plant 14
2.7.2. Stage 1: Establishment of aseptic culture 15
2.7.3. Stage 2: Shoot induction and multiplication of propagates 18
2.7.4. Stage 3*:* Rooting of plantlets 19
2.7.6. Stage 4: Acclimatization 20
2.8. Culture Media Composition 22
2.8.1. Macronutrient 22
2.8.2. Micronutrient 23
2.8.3. Organic supplements 24
2.8.4. Carbon and energy sources 24
2.8.5. Plant growth regulators 25
2.8.6. Gelling agent 26
2.8.7. P^H 27

Table of Contents (Continued)

3. MATERIALS AND METHODS 28
3.1. Experimental Site 28
3.2. Plant Material 28
3.3. Experimental Layout 28
3.3.1. Experiment I: Effect of sterilant chemical concentration and exposure time on the contamination rates of vanilla explants 28
3.3.2. Experiment II: The effects of combinations and concentrations of BAP and NAA on growth and development of vanilla shoots *in vitro* 30
3.3.3. Experiment III: Effect of different concentrations of IAA for best *in vitro* root initiation of vanilla shoots on different strengths of MS basal medium 31
3.3.4. Acclimatization 31
3.4. Data Collected 32
3.5. Statistical Analysis 34
4. RESULTS AND DISCUSSION 35
4.1. The Effect of Sterilant Chemical Concentrations and Exposure Time on Disinfection of Vanilla Explants 35
4.2. The Effects of Combinations and Concentrations of BAP and NAA on Growth and Development of Vanilla Shoots *In vitro* 39
4.3. The Effect of Different Concentrations of IAA and MS Basal Medium Strengths on the *In vitro* Root Initiation of Vanilla Shoots 46
4.4. Acclimatization of *In vitro* Derived Vanilla Plantlets 49
5. Summary and Conclusion 51
6. REFERENCES 55
7. APPENDICES 65

LIST OF TABLES

Table **Page**

1. Mean square values for the effect of different concentrations of sterilizing chemicals (*Berekina*®) and treatment exposure time on contamination rates of vanilla explants at JARC (2010)..... 35
2. Effect of sterilant chemical concentrations and durations of treatment on the contamination rates of vanilla nodal explants after 3 weeks of *in vitro* culture..... 37
3. Mean square values for the effect of BAP concentrations combined with NAA concentrations on varied shoot growth parameters of vanilla plantlets *in vitro* at JARC (2010)..... 40
4. The interaction effect of different levels of BAP and NAA treatments on *in vitro* shoot multiplication of vanilla after five weeks of culture at JARC (2010) 42
5. Correlation between different growth and development parameters of vanilla during *in vitro* shoot multiplication at JARC (2010)..... 43
6. Mean square values for the effect of different concentrations of IAA & MS basal medium strengths on the *in vitro* root parameters of vanilla after four weeks of culture at JARC (2010)..... 46
7. The interaction effect of different levels of IAA and MS salt strengths on the *in vitro* rooting of vanilla plantlets after four weeks of culture at JARC (2010) 47

LIST OF FIGURES

Figure **Page**

1. Aseptic culture of vanilla explants for initiation 30
2. Contamination types observed on *in vitro* culture initiation of vanilla explants. 38
3. Vanilla micro-shoots at different ages on multiplication media 45
4. Acclimatization of *in vitro* raised vanilla plantlets..... 50

LIST OF APPENDEX TABLES

Appendix Table **page**

1. Correlation coefficients among chemical concentration, duration of exposure and contamination rate for sterilization experiment of vanilla explants at JARC (2010) 66
2. MS media composition for *in vitro* culture experiments 66

LIST OF ABBREVIATIONS

BAP	6-Benzylamino Purine
Cm	Centimeter
CRD	Completely Randomized Design
EIAR	Ethiopian Institute of Agricultural Research
FAO	Food and Agriculture Organization
FAO STAT	Food and Agriculture Organization of the United Nations Statistical Database
ha	Hectare
HCl	Hydrogen chloride
IAEA	International Atomic Energy Agency
IAA	Indol -3-Acetic Acid
IBA	Indol -3-Butyric Acid
IISR	Indian Institute of Spices Research
JARC	Jimma Agricultural Research Center
MoE	Ministry Of Education
MS	Murashige and Skoog
NAA	Naphthalene Acetic Acid
NaOH	Sodium Hydroxide
NPK	Nitrogen Phosphorus and Potassium
PAA	Phenyl Acetic Acid
PGRs	Plant Growth Regulators
REGWQ	Ryan, Elinot, Gabriel, and Welsh Test
RS	Rimler-Shots
TNSRC	Tepi National Spices Research Center
UEPB	Uganda Export Promotion Board
USD	United States Dollar

PROTOCOL DEVELOPMENT FOR MICRPROPAGATION OF VANILLA (*Vanilla planifolia* Andr.) CLONE (Van.2/05)

ABSTRACT

Vanilla (Vanilla planifolia Andr.) is an important spice, which is economically valued for its cured fragrant beans that make one of the most expensive spices in the world trade. Although the crop had been introduced to Ethiopia and proved to adapt well to the hot humid agroecologies, the country did not exploit its potentials for vanilla production due to shortage of planting material; hence, the crop is still under maintenance at the Tepi National Spices Research Center (TNSRC). The crop is commonly propagated through stem cuttings. However, the technique is not recommended in modern vanilla cultivation, since it arrests subsequent plant growth and development and serves as an ideal means for the spread of varied diseases, etc. Therefore, there is a need of using the modern tissue culture techniques to alleviate the above stated challenges of multiplication. However, no efficient in vitro protocol had so far been developed to propagate the available vanilla clone in Ethiopia, which has been introduced recently from Mauritius. The present study was carried out with the main objective of developing micropropagation protocol for the V. planifolia accession (Van.2/05). In the current study, the initial materials for stock plant establishment were obtained from TNSRC, and established in the greenhouse of the Plant Biotechnology Laboratory of JARC, where all the subsequent experiments were carried out. Nodal explants and Murashige and Skoog (1962), MS, basal medium were used exclusively throughout the experiments. In all cases, the experiments were laid out in Completely Randomized Design (CRD) with factorial treatment combinations and replicated three times, whereby each replicate has 2 explants for hormone involving experiments. In this study, statistically highly significant ($p < 0.01$) differences were recorded from the use of different concentrations of active chlorine in local bleach (Berekina®) and varied levels of treatment durations on aseptic culture initiation. Therefore, the best result (72.23% rate of asepsis and survival) was obtained from the use of 20 minutes treatment of nodal explants using a solution Berekina® containing 5% active chlorine. With regard to shoot multiplication, the combined use of BAP and NAA revealed statistically very highly significant ($p < 0.001$) differences, whereby 2 mg l^{-1} BAP and 0.5 mg l^{-1} NAA proved to be the best providing the highest mean number (5.33) and length (4.9 cm) of shoots after five weeks of culture. Statistically very highly significant differences ($p < 0.001$) were also observed on in vitro rooting from the combined use of different MS basal medium strengths and IAA concentrations. Therefore, the best were recorded from the use of ½ MS basal medium added with 0.5 mg l^{-1} IAA for the number of roots and length, viz. 4.00 roots per plantlet with an average length of 6.1cm. Acclimatization of in vitro derived plantlets was also successful, whereby the average rate of ex-vitro survival was 83.4%. Therefore, the advent of this protocol could be of considerable value to relieve the problem of mass multiplication and enhance the expansion of vanilla cultivation in Ethiopia. And further studies on virus indexing, and cryopreservation should be considered using the protocol.

Keywords: *Berekina®, micropropagation, nodal explant, Vanilla planifolia*

1. INTRODUCTION

Vanilla belongs to the family *Orchidaceae*, of which some 110 species are reported so far **(**Talubnak and Soytong, 2010). Currently, only three of these species are under commercial production, i.e. *Vanilla planifolia* Andr. (Mexican Vanilla), *Vanilla pompona* Schiede (West Indian Vanilla) and *Vanilla tahitensis* (Tahitian Vanilla) (Uchida, 2010). However, *V. planifolia* Andr. also called vanilla fragrance, is the most popular of these three cultivated species with regard to its area of production, productivity and product quality, as well.

Vanilla planifolia Andr. is a diploid species with 2n = 32 chromosomes (Purseglove *et al.*, 1981), which is originated from the dense tropical forests of Mexico and Central America, and had been domesticated and cultivated in the last 1000 years. However, Mexico is reported to have the largest genetic diversity of *V. planifolia* as compared to other vanilla-producing countries (Gonzalez-Arnao *et al*., 2009). It was during the 18th century that vanilla vine cuttings were taken out of Mexico and introduced to Europe and other parts of the world (Raghavan, 2007). It is herbaceous perennial climbing terrestrial vine adapted to the warm humid tropics (Martins, 2008).

Vanilla is the only spice plant from the family *Orchidaceae* that is economically valued for its cured fragrant beans, which make one of the most expensive spices in the international trade, second to saffron (Giridhar *et al.,* 2001). Being one of the world's most popular flavoring material, vanilla finds extensive applications in confectionery and perfumery industries, thus it is among the top most marketed flavoring spices in the world (Correia *et al.,* 2009).

Currently, the crop is mainly cultivated for its highly aromatic pods or beans at different corners of the world. A total of 82,098 ha of land had come under vanilla cultivation in the year 2009, which is expected to increase its bean production by about 9,065 tones starting from 2009 (FAOSTAT, 2010).And total vanilla production in Africa found to be 2970 tones in the same year(2009) with 110.4kg/ha of productivity. However, in Ethiopia vanilla is not under cultivation rather it is under adaptation trial at Tepi Research Field and its adjoining areas.

Predominantly, vanilla is generally propagated through the vegetative means that commonly uses cuttings collected from a healthy and vigorously growing mother plant (Palama *et al.,* 2010). The length of the cuttings used for vegetative propagation of vanilla is commonly determined by the availability of mother plants. Normally, cuttings of 90 - 100 cm length are preferred for propagation, so as to shorten the crop's juvenile period, and shorten its maturity time and enhance flowering by a period of one or two years. However, if one uses shorter inter-nodal cuttings of about 20cm for propagation, as it is done in cases where there is shortage of planting material, the crop requires at least three years to attain maturity (McMahon, 2005).

So far, three accessions of *V. planifolia* had been introduced to Ethiopia at different times, and are currently under adaptation trial at the TNSRC. The first of these accessions (Van.1/93) was introduced from Indonesia, Bali Island, in 1993, while the second accession (Van.3/04) was obtained from Uganda in 2004, and the third accession (Van.2/05) was from Mauritius in 2005. Among these three accessions, available information from its origin had confirmed that the third to be the best commercial cultivar, both in its productivity and quality (Girma, 2010, Personal communication at TNSRC). This particular clone had also adapted well under Tepi and its surrounding agroecologies (JARC, 2008).

Shortage of planting material is the critical bottleneck that hampered wider dissemination and cultivation of the crop in Ethiopia. Therefore, this particular clone is among the top prioritized crop species for rapid mass multiplication using the modern tissue culture technique by the National Spices Research Program in particular, and the nation in general, (EIAR, 2009). Therefore, any work targeting to resolve the prevailing shortage of planting materials would in turn help a lot to respond to the national strategic plan that targets enhancing diversification of the national export commodities (Girma *et al.,* 2008).

In Ethiopia like any other developing country, where capital is a limiting factor while labor and land are found in abundance, the competitive advantage of the country in the world market will be supplying market standard agricultural products. In line with this, availability of favorable environment and colossal local and international market potentials had prompted large scale production of high value commodities, like spices, e.g.

vanilla, in the country for some time now. Hence, it has been long since investors started knocking the doors of the TNSRC, which is the center of excellence and national coordinating centre for spices research, thereby the mandated institution to supply planting material of elite varieties together with improved technology packages for the production of vanilla (Girma *et al*., 2008). However, the major setback to respond to the prevailing demand is associated with the inability of the center to produce sufficient quantities of planting materials using the conventional vegetative propagation technique, out of the handful number of vines found at its backyard (Zerihun *et al*., 2009).

Therefore, up scaling of vanilla production to a commercial level would face difficulties in Ethiopia at this moment, due to the inefficiency of the conventional vegetative propagation technique currently employed. This is mainly attributed to the limited number of stock plants the center has at hand that makes collection of sufficient quantities of stem-cuttings from the existing few mother plants nearly impossible, as it would result in retarded plant growth and development. Therefore, in order to respond to the prevailing demand for propagules of elite materials, the rapid regeneration of such specific clones through modern techniques, like tissue culture, becomes imperative (Kalimuthu *et al.*, 2006). Consequently, adoption of the modern micropropagation technique with optimum subculture stages enables production of millions of plantlets from a single mother plant within a short period of time becomes so vital (Shah *et al.*, 2008). However, effective exploitation of the technique requires development and/or optimization of the most efficient procedures at all stages of the process that suit the specific species and clone at hand.

Zerihun and his co-workers (2009) had reported vanilla micropropagation protocol using MS medium supplemented with 2mg/l BAP and 0.5mg/l kinetin and 3% sucrose as carbon source with success of 4.17 shoots per explant using Van.1/93 vanilla clone introduced from Indonesia in 1993.

To this end, no standardized protocol had been availed so far for efficient micropropagation of this vanilla clone (Van.2/05), which had been introduced recently from Mauritius. Thus, it became crucial to develop protocol for the rapid mass propagation and dissemination of this potential vanilla clone to exploit effectively the existing agroecologic potentials for vanilla cultivation in Ethiopia, thereby benefiting the nation from both import substitution and export of this invaluable spice, and enhance the foreign

currency gains of the country. Therefore, this research was conducted with the following objectives:

- To determine the optimum concentration of local bleach (*Berekina*®) solution and duration of exposure of vanilla nodal explants for successful sterilization

- To determine the effects of combinations and concentrations of BAP and NAA on multiplication (growth and development)of vanilla shoots *in vitro*

- To test the different concentrations of IAA for best *in vitro* root initiation of vanilla shoots using different strengths of MS basal media

2. LITERATURE REVIEW

2.1. Origin and Distribution of Vanilla

The genus vanilla is widely distributed across the tropics, with different species found spreading in the Central and Southern America as well as tropical Africa. Currently, three species of vanilla are known for their economic importance across the world, i.e. *V. planifolia*, *V. pompona* and *V. tahitensis*, of which the first one being by far the most important commercial species that is widely cultivated in regions situated between latitudes of 20° N and S of the equator (Martins, 2008).

Vanilla planifolia Andr. Syn. *V. fragrans* is originated in the Central American region, particularly around Mexico and first used by the Aztecs as a flavoring agent in their chocolate drinks (Correia *et al*., 2009). The crop was domesticated in its area of origin, Mexico, since the 18th century, followed by its introduction through vine cuttings to Europe (Anilkumar, 2004). Then after, it was taken to the areas around the Indian Ocean, Asia and Africa in the early 19th century (Bory *et al.*, 2008). The cultivated *V. planifolia* is a typical example of a crop species that had been disseminated from its area of origin, where it has its natural pollinators, to a completely new environment that lacks them.

2.2. Biology of Vanilla

Vanilla is a tropical climbing orchid belonging to the *Orchidaceae* family. It is a diploid plant with a chromosome number of 2n = 32 (Purseglove *et al*., 1981). According to Raghavan (2007), the name vanilla was derived from the Spanish word, vainilla, meaning “little pod”. The crop is a volubilate monocotyledon, fleshy, herbaceous perennial terrestrial orchid adapted to the warm humid tropics (Cassidy, 2010). Vanilla is an evergreen vine that often climbs to the top of trees. It has thick, oblong, 6 to 9 inch, pale to dark-green fleshy leaves arranged in an alternating fashion along the main stem. Similar to varied members of the family, vanilla produces aerial roots opposite to its leaves by which it clings to its support (Martins, 2008).

Vanilla (*Vanilla planifolia*) is a monoecious plant, whose flowers are borne at the very tips of the growing auxiliary liana branches (Anilkumar, 2004). Unlike many orchids, the flowers of vanilla last at most for a day (Purseglove *et al*., 1981). Naturally, vanilla flowers are not of a self-pollinated type; therefore, the plant requires pollination with the involvement of an external agent in order to set fruit. This is attributed to the presence of a natural barrier called rostellum (a specialized structure that separates the anther and stigma in the flower) that obstructs the free movement of pollen to the stigma in the flower (Maruenda and Christenson, 2010). Therefore, even for an individual vanilla flower to be selfed, an external pollinator or pollination agent is essential, either to remove the barrier, or to carry and transfer the pollen to the sticky stigma (Bsc, 2007).

On the other hand, it is only in their original home, Mexico, that vanilla flowers are pollinated with the help of their natural pollinator, the Melipona bee (*Melipona beecheii*) bee, which is endemic to that particular area (Martins, 2008). As a result, most cultivated vanilla plants elsewhere in the world are pollinated artificially through human intervention, i.e. by hand, that requires involvement of much labor. Consequently, this is one of the primary factors that contribute to the costliness of the spice in the world market. Therefore, growers are usually expected to inspect their plantations every day for open flowers so as to assign workers on time to undertake pollination (Klein, 2006).

In almost all of the cases, the crop is propagated through stem cuttings (Azeez, 2008). However, this method of propagation has several shortfalls, including being uneconomical and time consuming, as well as its side effect of arresting growth, development and yield, all of which resulting from collection of cuttings from the mother plants. Therefore, the conventional vanilla propagation using cuttings is reported to be inefficient and ineffective to address the prevailing huge demand for quantity and quality planting materials of vanilla, thereby calling for the adoption of modern rapid multiplication techniques (Kalimuthu *et al*., 2006).

2.3. Ecology and Agronomy of Vanilla

Vanilla (*V. planifolia)* can grow up to 1500masl in the humid tropical climates having a well-distributed mean annual rainfall of 1500 - 3000mm, but without prolonged dry periods. A warm humid climate with average temperatures in the range of 25°C - 32°C is

highly preferred for its cultivation (Anilkumar, 2004).Since it is a plant of forest undergrowth, the crop does well under microclimates with 50% shade, where it attains better growth and good production (Correia *et al*., 2009).

Vanilla can grow on any soil type provided it is well drained (Thomas *et al*., 2000). The crop prefers gravelly or sandy loam soils that are rich in humus, however, the most suitable soil for vanilla cultivation is lime stone origin having pH values of 6 to 7 (Ranadive, 2003). The vanilla plant is a surface feeder, having ramified roots in the top 2 to 5cm of the soil surface (Mudaliar and Ucuboi, 2005). Therefore, it is always essential to use a mulch cover after field transplanting so as to provide the plant with the most ideal soil environment that enhance root growth and development (Magala, 2002). Therefore, care should be taken not to injure the roots, while cultivating the field. Besides, it is also essential to ensure their deep rootedness while selecting support trees for the vanilla plant.

The combined use of 40 - 60g of N (nitrogen), 20 - 30g of P (phosphorus) and 60 - 100g of K (potassium) per vine per year is essential for successful growth and production of vanilla (Thomas *et al*., 2000). Besides, vanilla is also reported to respond well to foliar feeding with a 1% solution of 17:17:17 NPK soluble composite fertilizer that is sprayed once a month to boost plant growth and flowering (Horticultural College and Research Institute and Tamil Nadu Agricultural University, 2004).

Vanilla is particularly suitable as an intercrop in a diversified agroforestry farming system, and can easily be integrated into the systems of cacao, oil palm and coffee plantations. Combinations with avocado (*Persea americana*), tea (*Camellia sinensis*), Jack fruit (*Artocarpus heterophylus*) and many other perennial plant species with similar eco-physiological requirements are also possible (Augstburger *et al*., 2000).

2.4. Production, Uses and Marketing of Vanilla

Production of vanilla in the world is increasing from time to time to meet the ever increasing international demand for vanilla beans. The overall total area of land devoted to vanilla production in the year 2009 was reported to be 82,098 ha and the annual world production of its beans was also estimated to reach as high as 9,065 tones (FAOSTAT, 2010). The major producers of vanilla beans in the world are Madagascar, Indonesia,

Comoros, Uganda, India, Papua New Guinea, Mexico, China, French Polynesia, Tonga, Jamaica, Reunion Island, Mauritius, Mayotte, Costa Rica, Vanuatu and Fiji (Koekoek, 2005).

Vanilla is the world's most popular flavoring agent, and is among the most valuable spice crops in terms of cash return. The only true source of natural vanilla are the cured fruits of three obligatorily hand-pollinated and clonally propagated orchid species, viz. "Mexican vanilla" (*V. planifolia*), "Tahitian vanilla" (*V. tahitensis*) and "West Indian vanilla" (*V. pompon*) (Bory *et al*., 2008; Uchida, 2010). Vanilla beans are commonly used to flavor varied food products, liquors and perfumes; however, it is highly praised for its flavoring excellence in ice creams, soft drinks, chocolates, candy, tobacco, baked foods and pharmaceutical products (Giridhar and Ravishanker, 2004). Besides, it is widely used in diffusing the foul smell of rubber during tyre making (Uganda Export Promotion Board: UEPB, 2005).

Subsequent to nine months of maturation after anthesis, the vanilla beans should undergo elaborated processing procedures known as curing. Basically, curing involves a sort of fermentation process arising from naturally induced enzymatic actions at elevated temperatures, after which the beans will be dried and the flavor develops, freeing vanillin ($C_8H_80_3$), which is the most important flavoring compound within the vanilla beans (Bsc, 2007). In general, vanilla beans are reported to contain over 200 compounds, of which at least 25 are stated to be responsible for the overall aroma profile of vanilla, although vanillin is the most important of all (Sharp, 2009). The concentration of each component in the processed vanilla bean could principally vary with region, where the beans are produced.

The present international demand for vanilla is about 19,000 tons per annum. Practically, all exports are from the four major producing countries, with Madagascar being the uncontested leader contributing for 63% of the international supply in 2001. The contribution of Indonesia and the Comoros to the international market during this same period was reported to be 21% and 9%, respectively. The rest percentage of production is contributed by Uganda, and other modest suppliers like India, Jamaica and Papua New Guinea (Ghazali, 2006).

The international market prices of vanilla are highly volatile, due to the prevailing inconsistent supply and demand situations (Metzel and Stryker, 1994). Although the natural vanilla product is still preferred by many consumers and consumers are ready to pay for it (Correia *et al*., 2009), some are also using synthetic vanillin, mainly due to the prevailing constant raise in the prices of natural vanilla and its shortage of supply.

The major markets for natural vanilla exist in the USA, Canada, European Community, and other European countries, Japan, Australia, New Zealand and China. The total average annual world market demand of vanilla ranges from 2,000 to 3,000 metric tons. However, the international market for natural vanilla is consistently expanding due to its increasing use in the food industries. Therefore, there is a very high potential to expand its cultivation in the future, even into new areas where it is not among the traditional crops produced (Investment in Developing Export Agriculture: IDEA, 2000). On the other hand, the variation in the annual supply of natural vanilla to the international market is also affected by varied abiotic factors associated with its production, mainly prevailing climatic conditions, especially the off season rain fall. Besides, diseases and pests are also known to reduce the expected annual production significantly. In general, the overall international demand for vanilla is increasing at a greater rate than its supply (Koekoek, 2005). For instance, the average price of natural vanillin extracted from vanilla pods in the 2007 was reported to be in the range of 1,200 to 4,000 USD per kg, in contrast to the price of the synthetic vanillin, which costs less than 15 USD per kg (Hansen *et al*., 2009).

2.5. Constraints to Vanilla Production

Being a new crop to most of its current growing regions, the cultivation of vanilla faces several constraints, which include the problem of narrow genetic base that limits crop improvement, together with inadequate research attention given to the crop that will restrict the efficiency in responding to the problems associated with its production and post harvest handling, as well. However, the presence of ideal agroecologic conditions, including suitable altitude and ideal climate (sunlight, rainfall, humidity and temperature), together with the presence of conducive soil and water conditions (soil moisture status and irrigation potentials, as well as soil physical and chemical properties, etc.) highly encourages producers to expand their cultivated areas (Puthur, 2005).

Amongst the problems mentioned above, vanilla cultivation is seriously hampered by the incidence of several diseases. Conditions like excess soil moisture accumulation associated with insufficient drainage, the use of heavy mulch and too much shade are known to favor disease development. The vanilla plant is highly susceptible to many fungal and viral diseases. Among the diseases of fungal origin, the most prevalent ones include foot rot and wilting caused by *Phytophthora meadii, Fusarium oxysporum*, *Calospora vanillae, Sclerotium* rot, leaf rot, blights and brown spots or anthracnose that are caused by *Colletotrichum gloeosporioides*, etc. (Augstburger *et al.*, 2000).

As compared to most other crops, vanilla production is characterized with its labor-intensive and time consuming nature. Since natural pollination is so rare in vanilla, due to restrictive flower morphology and the absence of natural pollinators in most of the major growing regions, hand pollination is an indispensable activity to obtain the vanilla beans (Villanueva *et al.*, 2005). On of top this the post-harvest handling activities of vanilla pods (killing ,sweating, slow drying and conditioning of the vanilla beans) involve other artful processes that require a good deal of skill and experience (Correia *et al.*, 2009).

On the other hand, vanilla seeds are small sized by nature, which results in their poor germination potential. This is ascribed to the minimum quantities of stored food reserve found within their endosperm, which is so critical to support seed germination after prolonged storage (Porras-Alfaro and Bayman, 2007). Therefore, it is always essential to ensure successful association of the germinating vanilla seeds with mycorrhizal fungi, so as to gain better germination, as the latter would enable them to absorb, translocate and use the fungal carbohydrates trehalose (disaccharide sugar), as well as glucose additionally, in the course of their germination (Smith, 1973).

Conventionally, vanilla plants are propagated by stem cuttings, which are known to cause injury to the mother plant, besides perpetuating fungal, bacterial, as well as viral infections (Palama *et al.*, 2010). So, regeneration and *in vitro* mass multiplication are vital alternatives to minimize these problems, as well as to supply sufficient quantities of quality planting materials from a desired elite clone within a short period of time.

2.6. Plant Tissue Culture and Its Application

Plant tissue culture refers to the growing and multiplication of cells, tissues and organs on defined solid or liquid medium under aseptic condition and well controlled environment (Akin-Idowu *et al.*, 2009). The technology had been successfully used for large scale commercial production of and supply of true-to-type, pathogen-free plants of varied plant genotypes and species (Tsay, 2002), germplasm conservation of rare and endangered species, production of secondary metabolites, as well as rapid and large-scale multiplication of elite genotypes (Baskaran and Jayabalan, 2005). It also serves as an important tool for genetic manipulation, protoplast fusion, embryo rescue and other modern procedures that enhance plant improvement and other basic researches (Van, 2009).

The commercial application of this technology is primarily related to micropropagation, in which rapid proliferation of a selected clone is achieved either from tiny stem cuttings, or axillary buds, or somatic embryos and/or cell clumps in suspension cultures or bioreactors (International Atomic Energy Agency: IAEA, 2004). In the past, plant tissue culture techniques had been used in academic investigations of cellular totipotency and the roles of hormones in cyto-differentiation and organogenesis. Currently, tissue-cultured plants that have been genetically engineered provide a very good insight into plant molecular biology and gene regulation, beside their unparalleled benefits for plant improvement (Saito, 1993). Plant tissue culture techniques are also central to innovative areas of applied plant sciences, including plant biotechnology and agriculture (Mineo, 1990). The first commercial use of plant tissue culture on artificial media was applied in the germination and growth of orchids in the 1920's (Van, 2009).

Micropropagation is the application of modern tissue culture technology for mass propagation of economically important plant species (Venkateswarlu and Korwar, 2005). It offers an alternative to the conventional vegetative propagation system and is mainly aimed at enhancing the rate of multiplication (Govinden-Soulange, 2009). Plants can be multiplied *in vitro* on artificial media, under controlled environment, irrespective of seasonal variations and weather, on a year-round basis (IAEA, 2004). Production of high quality and healthy planting materials of ornamentals, forest trees, and fruit tree species propagated from vegetative parts, has created new opportunities in global trading for

producers, farmers, and nursery owners, and had also contributed a lot for rural employment (Yesil-Celiktas, 2010).

So far, vanilla micropropagation had been carried out following different paths, including i) shoot bud proliferation, ii) adventitious shoot production, iii) meristem and/or nodal culture, as well as iv) somatic embryogenesis (Venkateswarlu and Korwa, 2005). Moreover, protoplast fusion had also been successfully carried out between ***V. planifolia*** and *V. andamanica* to produce a hetrokaryon and was helpful to transfer important traits to the former (Divakaran *et al*., 2008).

Different workers had succeeded in micropropagating vanilla through axillary buds multiplication by culturing shoot tips, root tips or stem nodes. Besides, prominent works had also been carried out on vanilla tissue culture through mass production of protocorm like bodies (PLBs) as well as somatic embryogenesis. However, mass production of *V. planifolia* through indirect shoot organogenesis is generally rare, and there are numerous biological unknowns about the induction of regenerants following this morphogenetic pathway (Palama *et al.,* 2010).And also this technique may expose plantlets to develop somaclonal variation.

The primary advantage of micropropagation is to mass-produce genetically identical, physiologically uniform, developmentally normal and pathogen-free plantlets that have higher *ex-vitro* survival, and at a reasonable cost (Bhojwani, 2005). To this end, Geetha and Shetty (2000) had reported their success in micropropagating *V. planifolia* plantlets from shoot tip and nodal segment explants. The explants were cultured on MS basal medium added with 6-Benzylamino purine (BAP) (1 mg 1^{-1}) for 10 weeks for initiation. The proliferating clusters were then sub-cultured at a two to three weeks interval onto Nitsch and Nitsch, 1969 (N-69) basal medium fortified with BAP (0.5 mg 1^{-1}), d-biotin (0.05 mg 1^{-1}), folic acid (0.5 mg 1^{-1}) and 2% sucrose for shoot elongation, whereby culture media were refreshed every 2 to 3 weeks. This procedure had yielded 9 plantlets per explant of 7 to 8 cm length shoots that were later acclimatized under poly tunnels successfully.

On the other hand, Giridhar *et al*. (2001) had also reported their success on *in vitro* propagation of *V. planifolia* through axillary bud proliferation making use of shoot tip and nodal explants. Later on, the *in vitro* shoots were used as a source of nodal explants to pursue enhanced shoot multiplication on MS medium supplemented with BAP (1.0 mg 1^{-1}), NAA (0.2 mg 1^{-1}) and sucrose (3%). In addition, ingredients like thiamine HCl (5.0 mg 1^{-1}), glycine (0.25 mg 1^{-1}), myo-inositol (1000 mg 1^{-1}), casein-HCl (500 mg 1^{-1}), as well as $AgNO_3$ (3.4 mg 1^{-1}) were also added to the medium to promote the rate of shoot multiplication. The procedure was reported to yield the longest shoot and 5.4 shoot numbers per explant.

In subsequent years, Giridhar *et al*. (2003) had used another *in vitro* procedure for micropropagating *V. planifolia*, whereby BAP (5.0 mg 1^{-1}) and Phenyl Acetic Acid (PAA) (1 mg 1^{-1}) were used. Therefore, they were able to produce an average of 2-3 shoots having an average length of 2.32 cm, after 30 days of culture. However, the protocol proved to be less efficient than their earlier procedure (Giridhar *et al.*, 2001) for mass propagation of this particular species. This was ascribed to the response differences of BAP when combined with that of NAA or PAA, the latter being a relatively weaker type of auxin to boost the efficiency of BAP (George *et al.*, 2007). On the other hand, BAP was reported to be among the best growth regulators to induce multiple shoots of *V. planifolia* at concentrations ranging from 1.0 - 3.0 mg/l (Indian Institute of Spices Research: IISR, 2005).

From the above three experiments, the first two had used different media compositions for shoot multiplication and elongation, which could show significant variations in their respective costs of production. This signifies the need for a simple and efficient *in vitro* protocol involving a single medium composition for both multiplication and elongation of *V. planifolia* plantlets, since it reduces both time and cost of production simultaneously, and avoids any fear of somaclonal variation.

2.7. Sequential Stages of Micropropagation

In general, plant micropropagation has five distinct sequential phases/stages, which include the stage of mother plant selection and preparation, stage of aseptic culture establishment, multiplication, elongation and/or rooting, as well as *ex-vitro* acclimatization and hardening-

off (Panhwar, 2005). Unlike the conventional vegetative propagation, micropropagation is relatively costlier, which of course can be compensated with its varied unique benefits mentioned above, as compared to the former. Therefore, optimization of protocols for the development of simplified automated environmental control systems and improvement of the *in vitro* culture systems are extremely essential to bring about significant cost reductions on plantlets derived from tissue culture (Bhojwani, 2005).

2.7.1. Stage 0: Preparation of stock plant

Before micropropagation commences, careful attention should be given to the selection of stock plants. They must be typical of the variety or species, and free from any symptoms of disease. It may be advantageous to treat the chosen plant in some way to make *in vitro* culture successful (Georg *et al.,* 2007). Growth, morphogenesis and rates of propagation *in vitro* can be improved by appropriate environmental and chemical pre-treatment of stock plants (IAEA, 2004).

In vitro cultures are initiated explants, which theoretically are taken from any part of the plant. However, the success of their establishment *in vitro* varies with their sources and plant species under consideration (Yesil-Celiktas, 2010). The most common types of explants that are used in commercial plant micropropagation include shoot-tips, meristem-tips, nodal segments and/or axillary buds, in which case the later will consist a piece of stem possessing axillary buds (George *et al*., 2007). In some plants, leaf discs (tomato, Sorghum and tobacco), intercalary meristems from nodes (Banana, Seaweeds), miniatures of stems, immature zygotic embryos (*Capsicum annuum* L., Canola) are also used as a source of explant to initiate *in vitro* cultures. To ensure success in the endeavor, the explant source or the stock plant, should be vigorously growing and true-to-type in conformity to the original variety, as well as clean and free from any disease causing pathogens (Jain and Ishii, 2003).

In general, as size of the explant decreases, the chance of getting contact with contaminants decreases consequently, their contamination rate decreases considerably (Knauss, 1979). On the other hand, explants collected from underground plant parts are usually found to be heavily loaded with microflora, and hence difficult to cleanse the explants as a result it demands stringent sterilization methods (Akin-Idowu *et al.,* 2009).

Likewise, explants obtained from aerial (above soil) plant parts could also be found heavily contaminated with microorganisms, although the microbes would not form tight associations with the plant tissue, hence making the disinfection task relatively easier, provided that the stock plants are maintained well protected in the greenhouse (Jain and Ishii, 2003).

Among others, nodal culture is reported to be of significant value for micropropagating *V. planifolia* giving rise to better elongated shoots in culture, hence making it highly popular for commercial propagation of the species. Nodal explants are highly preferred in vanilla micropropagation, since they can provide sufficient quantities of elongated shoots having a number of nodal segments for successive sub culturing. Apart from this, nodal explants are suitable to have effective cultures for mass multiplication of *in vitro* shoots, rooting as well as *ex vitro* acclimatization, on top of its benefits to avert somaclonal variations (George and Ravishankar, 1997).

2.7.2. Stage 1: Establishment of aseptic culture

A good clean explant, once established in an aseptic condition, can be multiplied several times; hence, explant initiation in an aseptic condition should be regarded as a critical step in micropropagation (George *et al.,* 2007). Microbial contamination presents a major challenge to the initiation and maintenance of viable *in vitro* cultures. Contamination in this paper refers to the infection of an explant by either fungi, bacteria, and/or yeasts (Bausher and Niedz, 1998), which are commonly found on the surfaces and in the natural openings of the explant material, and which become manifested in the course of culture initiation and can either be overt or covert (IAEA, 2004). There are several possible sources of contamination in the course of *in vitro* culture, which include the explant material, the culture vessels, the medium itself, the instrument used to handle the explant, etc. (Odutayo, 2007). In general, the explant surface carries a wide range of microbial contaminants, therefore it is imperative to use appropriate techniques to sterilize the explants prior to inoculation.

Plant tissue culture media which are rich in sucrose and other organic nutrients readily support the growth of different microorganisms, including bacteria and fungi, which can grow relatively much faster than the cultured tissues on the medium, thereby killing the

tissues at the end (Rai, 2007). Therefore, sterilization of explants is a key step in any plant tissue culture work, as the removal of all microorganisms is essential to attain successful initiation, growth and development of the cultured tissues *in vitro*, which otherwise would be overwhelmed by the contaminants (Carrel, 1923).

In the course of *in vitro* culture contamination of explants is a function of several plant and environmental related factors, such as plant species, age of the stock plant, as well as the type and position of the explant, together with the prevailing weather condition and the growth condition to which the stock plants were exposed prior to their collection (Sulaiman, 2004). In this regard, the problem of microbial contamination becomes further exacerbating when the explant material is sourced directly from field grown plants (Seymour *et al.*, 2003). Despite all the efforts made to select the best stock plant and ideal time of collection, it is almost impossible to avoid microbial contamination in the course of *in vitro* culture (Bauer and Johnston, 1999).

To reduce the level of microbial contamination of the stock plant and hence obtain axenic explants, various strategies have been and are still being employed in tissue culture labs. In this regard, the primary strategy is growing the stock plants under controlled conditions in the greenhouses, whereby they are supplied with proper nutrition, careful watering at their root base avoiding wetting of the foliage at any cost, together with their regular treatment with a contact and systemic antimicrobial chemicals. The second option is stated as raising the stock plants in the field, but with regular treatment using contact and systemic antimicrobial agents prior to collection of the explants. The third strategy involves pruning of donor plants grown in the field so that they could produce new shoot tips that would have minimal microbial loads, which in turn could give contaminant-free explants (Matthews and Duncan, 1993). Therefore, different workers had adopted the first strategy indicated above and used different types of explants from greenhouse grown healthy, well treated and actively growing stock plants of *V. planifolia*. Consequently, Geetha and Shetty (2000) and Kalimuthu *et al*. (2006) had used shoot tips and nodal explants for their micropropagation study of vanilla, while Janarthanam and Seshadri (2008) had used leaf segment and nodal explants. In line with this, Zerihun *et al.* (2009) had used greenhouse raised vanilla stock plants as a source of nodal explants in the course of their studies.

In general, regarding establishment of axenic cultures primary consideration is given to the selection of suitable sterilizing agents and determination of appropriate duration of explant exposure to the chemicals. As a result, the chemical selected should be of a type that could be easily removed from the explant surface through repeated rinsing (Panhwar, 2005), while the duration of exposure is sufficient enough to ensure higher rates of explant survival after treatment. However, as the use of some antimicrobial chemicals (such as antibiotics, and/or some carcinogenic chemicals like mercuric chloride) could possibly harm the users, on top of causing phytotoxicity, retarded explant growth, as well as encouraging resistance buildup for the chemicals, it is essential to take the utmost care while selecting the particular chemical for use.

On top of treating the explant material, tackling contamination in the course of *in vitro* culture requires appropriate care to make sure all other possible sources of contamination are cleansed, as well. Among all these sources, those associated with the culture medium, culture vessels and the instruments that are used to handle explants, etc., could be tackled through autoclaving at 15 psi and 121^0C for 15-20 minutes. Furthermore, the latter two could also be effectively cleansed through oven drying at 180^0 C for 3 hours. However, those sources of contamination associated with the explant, personal hygiene and/or the working environment require adoption of other control strategies (Srivastava *et al*., 2010).

According to Bhojwani and Razdan (1996), the problems of contaminants associated with explants could be resolved following proper aseptic procedures involving several steps. Therefore, the explants should be washed thoroughly initially using a suitable detergent and clean water, and left under a running tap water for several minutes prior to chemical sterilization. According to Oyebanji *et al.,* (2009), exposure of explants to 70% ethanol solution for 0.5 - 1.0 minute prior to their treatment with other sterilizing chemicals is a generally accepted procedure in sterilization. Hence, Balaji *et al*. (2008) had reported the use of ethanol (70%) for one minute followed by a treatment with either 0.1% mercuric chloride or 1.0% sodium hypochlorite solutions for 5 and 10 minutes, respectively as most effective for explant sterilization. However, the use of mercuric chloride is strongly discouraged in vanilla sterilization like the cases in other plant species due to its carcinogenic and high toxic nature, on top of its costliness.

Therefore, Zerihun and his co-workers (2009) had gently washed their nodal explants from green-house grown *V. planifolia* stock plants with clean water and a liquid detergent (Largo). Subsequently, they kept their explants under a running tap water for 30 min, prior to their soaking in a Kocide-101 solution (3 g 1^{-1}) for another 30 minutes followed by 3 - 5 times rinsing with sterilized distilled water. Then after, the explants were transferred to the laminar flow hood cabinet and dipped in a 70% alcohol solution for 5 minutes, and then rinsed 3 to 5 times with sterilized distilled water. Later, they were kept in sterile bottles containing $HgCl_2$ solution (0.1%) and gently shaken for 5 minutes, and were thoroughly rinsed with sterilized distilled water 3 - 5 times to completely get rid of the chemical residues from the tissue surface. The sterilized nodal segments were then laid on pre-autoclaved sterile Petri dishes to remove their bleached parts at both ends before inoculation on the conditioning medium for three days. The procedure had resulted in 90% contaminant free explants of which 60% fully survived from exposure effect of the steriliant chemical.

2.7.3. Stage 2: Shoot induction and multiplication of propagates

Initially this stage of micropropagation method is recognised by the formation, growth and proliferation of adventitious shoots from the primary explant. Subsequently at this stage subcultures might be established from individual shoots by the techniques familiar in shoot culture and results a highly proliferative shoot mass and a very rapid rate of Propagation (George *et al.*, 2007).

In vitro shoot multiplication could be attained either through axillary bud proliferation, or shoot elongation involving production of multiple nodes in cases where nodal segments could be used in the course of sub culturing (Baskaran and Jayabalan, 2005). In line with this, Misra *et al.*(2006) had tested the shoot multiplication response of different explants of *V. planifolia*, including nodes having axillary buds, internodes and leaf segments, on SH (Schenk and Hildebrandt, 1972) medium supplemented with different levels of BAP. Therefore, nodal segments having axillary buds were found to be the best explants for multiple shoot induction when cultured on SH medium added with BAP in the ranges of 1.0 – 3.0 mg 1^{-1}. However, higher concentrations of BAP were reported to be inhibitory for shoot multiplication or callus mediated shoot regeneration of vanilla (IISR, 2005).

On the other hand , George and Ravishankar (1997) had succeeded in clonal propagation of *V. planifolia* making use of axillary bud explants collected from field grown mother plants cultured on MS medium added with BAP (2 mg 1^{-1}) and NAA (1 mg 1^{-1}). Therefore, an average of 42 shoots were produced from a single axillary bud explant over a period of 134 days culture on solid MS medium supplemented with BAP (1 mg 1^{-1}) and NAA (0.5 mg l^{-1}), following 2-3 subcultures in liquid medium having a similar composition.

In another instance, Kalimuthu *et al*. (2006) had obtained an average of 9.43 shoots per vanilla explant using shoot tip and stem node explants cultured on semi solid MS basal medium added with BAP (1.0 mg 1^{-1}), coconut water, CW (150 ml 1^{-1}), and sucrose (30 g 1^{-1}). This protocol seems better than that of George and Ravishankar (1997), as it involves fewer types of media components, which could thus make the protocol less costly.

2.7.4. Stage 3*:* Rooting of plantlets

Success in micropropagation requires not only efficient shoot multiplication protocols, but also successful rooting of the *in vitro* produced plantlets (Sanchez *et al.*, 1996), which is influenced by different factors. Among all others, strength of the basal medium, the interaction between genotype and hormone composition, and the level of sucrose in the culture medium are reported to have a significant effect on *in vitro* root development of plantlets (Al-Khalifah, 2005). In most of the cases, tissue culturists use activated charcoal to promote or inhibit some kinds of *in vitro* growths, on top of its benefits for shoot quality and also enhanced root development, i.e. formation of numerous lateral roots and longer main roots (Pan and Jataden, 1999). In general, charcoal mainly employed in culture medium for the following main purposes, i.e. creation of a darker rooting environment, adsorption of undesirable/inhibitory substances, as well as growth regulators and other organic compounds that are carried over from previous sub culturing (Pan and Jataden ,1998).

Increasing the level of sucrose in the medium was also reported to enhance the contents of sugars and starch in the tissues, and hence the number of shoots and stomata; while decreasing the water potential and size of the stomata in the course of *in vitro* growth, all of which directly related with the survival efficiency of plantlets in the course of *ex vitro*

acclimatization (George *et al*., 2007). However, such situations are reported to hamper the photoautotrophic growth potentials (generation of leaves) of the *in vitro* plantlets (Jo, 2009). In contrast to this, the use of reduced sucrose level during *in vitro* culture under light condition was stated to enhance the number of leaves and roots, as well as size of the stomata, and photosynthetic nature of the plantlet in *Annona glabra* L. (Oliveira, 2008).

In most of the cases, auxins are commonly used to hasten *in vitro* rooting of plantlets, as they promote cell growth and elongation, as well as root differentiation (Mineo, 1990). The most common auxins like IBA, IAA and/or NAA, either alone or in combination, are reported to promote the *in vitro* root induction of plantlets (Shah, 2006); though they reveal differences in their action due to varied inherent reasons (Ahmad, 2004). Moreover, the combined use of auxins and cytokinins at equal ration promotes callus induction, however it promotes multiple shoot proliferation when the cytokinin level exceeds the auxin level (Iliev, 2010).

In line with this, Gopi *et al.* (2006) had used half strength MS basal medium added with 1.0 mg l^{-1} IAA and reported 90 % rooting success in vanilla, with an average root number and length of 3.5 and 6.0cm, respectively; which at subsequent acclimatization revealed 85% survival. Likewise, Gantait *et al.* (2009) had succeeded in inducing vanilla roots *in vitro* using full strength MS media supplemented with 0.25 mg l^{-1} IAA and 2 g l^{-1} activated charcoal. In another instance, Giridhar *et al.* (2001) had used Rimler-Shotts (1973), RS; basal medium supplemented with 2 mg l^{-1} IBA and obtained 100 % rooting efficiency with an average of 3.2 roots per explant of *V. planifolia*.

2.7.6. Stage 4: Acclimatization

Good quality propagules with well developed roots and leaves are easy to acclimatize to the external environment. Therefore, any efficient successful acclimatization protocol must ensure that the acclimatizing plantlets maintain active growth during their entire weaning period (Iliev, 2010). However, a substantial number of *in vitro* regenerated plantlets will not survive after their transfer *ex vitro* to the greenhouse or field environments, as the latter have substantially lower relative humidity, higher light intensity and less clean environment, which are stressful to the delicate *in vitro* derived plantlets. Therefore, most plantlets derived from tissue culture require a stage of

acclimatization and hardening-off, which is a final and decisive stage to succeed in micropropagation endeavour. Here, the *in vitro* derived plantlets will be subjected to appropriate weaning condition where they will gradually adapt themselves to the external environment, in order to maximize their field survival rate (Hazarika, 2003). However, in most of the cases this stage is given minimal attention, and hence a substantial number of *in vitro* regenerated plantlets are usually known to be lost.

This is mainly associated with the situation that *in vitro* propagated plants possess thin, soft, photosynthetically hypoactive leaves having less epicuticular wax and under developed and malfunctioning stomata that would result in excessive transpiration (Panhwar, 2005). In general, as plantlets are supplied with sucrose (or some other carbohydrate) in the course of their *in vitro* growth, and kept under lower light intensity, they are unable to photosynthesize their own food readily. Therefore, it is essential to provide them with a stimulus, which they were not supplied in their closed *in vitro* environment, to make them fully capable to produce their own carbon requirements and reduced nitrogen (i.e. before they become capable of feeding themselves - autotrophic) (Hazarika , 2003). Such changes will only take place after the plantlets have spent several days *ex vitro*.

Therefore, it is not surprising that the transfer of plantlets, whether rooted or not, from the tissue culture environment to the greenhouse results in tissue stress, and is often associated with slow growth and significant death of plantlets (Panhwar, 2005). Therefore, *in vitro* root development of plantlets is imperative as it commonly enhances the success of field transplanting in vanilla, since the production of functional roots would create a favourable plant-water balance under field condition. In this regard, Hapsoro and Yusnita (1997) had conducted an acclimatization experiment transferring micro-shoots of *V. planifolia* on to six different types of soil mixes, although all gave best results, i.e. 100% survival after field transplanting.

On the other hand, Zerihun and his co-workers (2009) have succeeded in acclimatizing *in vitro* derived plantlets of *V. planifolia* gaining a survival rate of more than 85%. In this particular case, the *in vitro* derived plantlets were retained in a poly-tunnel erected under a screen-house, at 100 % RH (Relative Humidity) at the initial stage that later was reduced

to 60% after two weeks, followed by a further reduction to the ambient RH within two more weeks time.

2.8. Culture Media Composition

The regulation of *in vitro* growth and development of plant tissues is a complicated process, which among others depends on the genetic makeup of the plant, the culture environment and the artificial media composition on which the plant tissues are placed to grow (Rai, 2007). Growth and development of plants be it under *ex-vitro* or *in vitro* conditions is highly determined by the nutrient composition of their growth medium, as plants cannot live and grow in the absence of water and required minerals (IAEA, 2004). Therefore, as any living tissue, plant material could only grow *in vitro* if are provided with appropriate medium, containing all the required mineral nutrients in appropriate proportion (Stone, 2006). A tissue culture medium usually contains inorganic salts, supplying the major and minor elements necessary for the growth of whole plants, together with varied types of vitamins and amino acids, though the last two could be optional in some cases (George *et al*., 2007).

2.8.1. Macronutrient

Macronutrients are groups of essential elements providing the six major elements-nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), and sulfur (S)-required for plant cell or tissue growth relatively in larger quantities(> 0.5uM) by the plant. The optimum concentration of each nutrient for achieving maximum growth rates varies considerably among species (PhytoTechnology Laboratories, 2003).

Nitrogen is essential to plant life. It is a constituent of both proteins and nucleic acids also occurs in chlorophyll and some coenzymes as a result the element required for plant growth and development in greatest amount. Plant cells may grow on nitrates alone, but considerably better results are obtained when the medium contains both a nitrate and ammonium nitrogen source. Certain species require ammonium or another source of reduced nitrogen for cell growth to occur. Nitrates are usually supplied in the range of 25-20mM; typical ammonium concentrations range between 2 and 20 mM. However,

ammonium concentrations in excess of 8mM may be deleterious to cell growth of certain species (Bhojwani and Razdan, 1996).

Phosphorus is vital for cell division as well as in storage and transfer of energy in plants. Its role in photosynthesis is also important. Too little phosphorus causes plants to be abnormal and sickly. Potassium is necessary for normal cell division, for synthesis of proteins, chlorophyll, and for nitrate reduction. The level of K^+ *in vitro* is rarely a problem but certain species are sensitive to high levels. Sulphur is present in some proteins. It is quite often present as an impurity in agar (Bhojwani and Razdan, 1996).

Calcium as calcium pectate is an integral part of the walls of plant cells and helps maintain integrity of the membrane. High levels of calcium have been shown to promote callose deposition thereby inhibiting cell extension. Found that stomata were more open in plants grown in the presence of high Ca^{2+} .Cytoplasmic Ca^{2+} is also involved in the regulation of hormone responses and mediates in responses to environmental factors such as temperature and light. Magnesium is a component of chlorophyll and a co-factor for many enzyme reactions. Magnesium uptake is not usually limited, except at low pH (George *et al.*, 2007).

2.8.2. Micronutrient

The majorities of the micro-elements are required in trace quantities (< 0.5uM) and quite often may get carried into the medium as impurities in other ingredients. They may also get carried-over with the explant or tissues and support growth for several weeks without showing any deficiency symptoms. This and the interaction amongst the microelements make the study of individual elements slightly complicated. The microelements are essential as catalysts for many biochemical reactions. Microelement deficiency symptoms include reduced lignification (Cu, Fe), rosetting (Zn, Mn), leaf chlorosis (Fe, Zn, Mn) and shoot tip necrosis (B). Certain elements, such as Co and Ni, can inhibit ethylene synthesis (PhytoTechnology Laboratories, 2008).

Iron is usually added as iron sulphate, although iron citrate can also be used. Ethylenediamine tetra acetic acid (EDTA) is usually used in conjunction with the iron

sulphate. The EDTA complexes with the iron so as to allow the slow and continuous release of iron into the medium. Uncomplexed iron can precipitate out of the medium as ferric oxide (George *et al.*, 2007).

2.8.3. Organic supplements

Growth and morphogenesis of plant tissue cultures can be improved by small amounts of some organic nutrients. These are mainly vitamins and amino acids. The amount of these substances required for successful culture varies with the species and genotype, and is probably a reflection of the synthetic capacity of the explant. To achieve the best growth of the tissue it is often essential to supplement the medium with one or more vitamins and amino acids. Various standard media show wide differences in their composition with respect to vitamins and amino acids (IAEA, 2004).Thiamine: amino acid synthesis and cofactor in carbohydrate metabolism. Pyridoxine and myo-inositol also added to the medium to stimulate cell growth and in tissue development (PhytoTechnology Laboratories, 2008).

Amino acids are also commonly included in the organic supplement. The most frequently used is glycine (arginine, asparagine, aspartic acid, alanine, glutamic acid, glutamine and proline are also used), but in many cases its inclusion is not essential. Amino acids provide a source of reduced nitrogen and, like ammonium ions; uptake causes acidification of the medium. Casein hydrolysate can be used as a relatively cheap source of a mix of amino acids and Cysteine included in media as antioxidant to control phenolic oxidation and hence blackening of explants (PhytoTechnology Laboratories, 2008).

2.8.4. Carbon and energy sources

Since *in vitro* growing plants are not completely autotrophic, they also require an external source of energy; therefore, addition of sucrose or any other source of carbon to the culture medium is mandatory (Silva, 2004).Sucrose is cheap, easily available, readily assimilated and relatively stable and is therefore the most commonly used carbon source. Other carbohydrates (such as glucose, maltose, galactose and sorbitol) can also be used, and in specialised circumstances may prove superior to sucrose (IAEA, 2004).

Sucrose concentrations of culture media normally range between 2 and 5 percent. Use of autoclaved fructose can be detrimental to cell growth. Carbohydrates must be supplied to the culture medium because few plant cell lines have been isolated that are fully autotropic, e.g., capable of supplying their own carbohydrate needs by CO_2 assimilation during photosynthesis (Mineo, 1990).

2.8.5. Plant growth regulators

An other important components of plant tissue culture medium that determine growth and development of the tissues *in vitro* are plant growth regulators (PGRs), i.e. a group of organic substances that usually required only in a very small concentration (Mineo, 1990). There are five main classes of plant growth regulator used in plant cell culture, viz; Cytokinins, Auxins, Gibberellins, Abscisic Acid and Ethylene (Muller, 2008).The first two have wider application in plant tissue culture practices.

Cytokinins promote axiliary shoot proliferation by decreasing the apical dominance of plants. The most frequently used cytokinins in plant tissue culture medium include BAP (6-benzylaminopurine), Kinetin, Zeatin and 2-iP (6-γ-γ-dimethylaminopurine), of which BAP and Kinetin are synthetic while the rest are naturally occurring types. As they are mostly heat stable, they can be added to the medium prior to autoclaving (Iliev, 2010). Among all the synthetic cytokinins, BA or BAP (Benzyladenin or Benzyl amino purine) and Kinetin are reported to have wider application in micropropagation technique since they can promote multiple shoot development, especially when used in combination with auxins (Liang, 2007). However, Zeatin and 2iP are not commonly used in tissue culture works, as they are expensive and relatively unstable (Cheesman, 2009).

Auxins promote both cell division and cell growth and the dominantly used auxins in the course of *in vitro* culture are NAA (α- Naphthalene Acetic Acid), 2, 4-D (2, 4-dichlorophenoxy acetic acid), IAA (Indole-3-acetic acid), IBA (Indole-3-butyric acid), and NOA (2-naphthyloxyacetic acid). Here also NAA, 2, 4-D, IBA and, NOA are synthetic auxins unlike IAA which are naturally occurring (Lack and Evans, 2001).

There are numerous, naturally occurring, structurally related compounds termed 'gibberellins'. They are involved in regulating cell elongation, and to break bud dormancy. On the other hand Abscisic acid (ABA) inhibits cell division. It is most commonly used in plant tissue culture to promote distinct developmental pathways such as somatic embryogenesis (Bhojwani and Razdan, 1996).

Ethylene is a gaseous, naturally occurring, plant growth regulator most commonly associated with controlling fruit ripening in climacteric fruits and its use in plant tissue culture is not widespread. It does, though, present a particular problem for plant tissue culture. Some plant cell cultures produce ethylene, which, if it builds up sufficiently, can inhibit the growth and development of the culture. The type of culture vessel used and its means of closure affect the gaseous exchange between the culture vessel and the outside atmosphere and thus the levels of ethylene present in the culture (George *et al.*, 2007).

2.8.6. Gelling agent

Media for plant cell culture *in vitro* can be used in either liquid or 'solid' forms, depending on the type of culture being grown. For any culture types that require the plant cells or tissues to be grown on the surface of the medium, it must be solidified (more correctly termed 'gelled'). Agar, produced from seaweed, is the most common type of gelling agent, and is ideal for routine applications (George *et al.*, 2007).

Agar has several advantages over other gelling agents. First, when agar is mixed with water, it forms a gel that melts at approximately 60°-100° C and solidifies at approximately 45°C; thus, agar gels are stable at all typical incubation temperatures. Additionally, agar gels do not react with media constituents and are not digested by plant enzymes (IAEA, 2004). The firmness of an agar gel is controlled by the concentration and brand of agar used in the culture medium and the pH of the medium. The agar concentrations commonly used in plant cell culture media range between 0.5% and 1.0%; these concentrations give a firm gel at the pH levels typical of plant cell culture media (Bhojwani and Razdan, 1996).

Another gelling agent commonly used for commercial as well as research purposes is gelrite (phythagel). This is a product of bacterial fermentation and should be used at 1.25-2.5 g/l, resulting in a clear gel which aids in detecting contamination. Alternative methods of support have included use of perforated cellophane, filter paper bridges, filter paper wicks, polyurethane foam, and polyester fleece. Whether explants grow best on agar or on other supporting agents varies from one species of plant to the next (IAEA, 2004).

2.8.7. P^H

The pH of a solution is a measure of the concentration of hydrogen ions in the solution. The pH scale extends from very acid (0) to very alkaline (14) with 7 being the "neutral" point. The pH of most culture media is adjusted to 5-6 before autoclaving. The pH can influence the solubility and availability of ions in nutrient media, the ability of agar to gel, and the subsequent growth of cells. P^H below 5 affects solidification of medium, P^H above gives fairly hard medium, pH changes affects uptake of nutrients. Thus accurate determination and control of media pH are necessary. Generally, pH is determined with a pH meter (IAEA, 2004).

3. MATERIALS AND METHODS

3.1. Experimental Site

The experiment was conducted under laboratory condition (Tissue Culture Laboratory) at JARC, which is located 363 km South West of Addis Ababa at 7°40'N latitude and 36°47'E longitudes. The Center has maximum and minimum temperature of 26.2^{0}C 11.3^{0}C, respectively and with 1529.5 mm average rain fall. The Research Centre, which is found under the umbrella of the Ethiopian Institute of Agricultural Research, specializes in agricultural research, which includes serving as National coordinating Center for Coffee Research (EIAR, 2004).

3.2. Plant Material

A recently introduced commercial vanilla (*Vanilla planifolia* Andr.) clone (Van. 2/05) was used in this study. The material was introduced from Mauritius in 2005 and is currently under adaptation trial at TNSRC. Fifty seedlings of the stated clone, propagated through the conventional vegetative cutting system, were obtained from TNSRC and maintained under the greenhouse condition of JARC, to be used as stock plants.

3.3. Experimental Layout

3.3.1. Experiment I: Effect of sterilant chemical concentration and exposure time on the contamination rates of vanilla explants

Young and actively growing stem nodes from the greenhouse maintained stock plants of the recently introduced *Vanilla planifolia* clone (Van. 2/05) were collected, and thoroughly washed with liquid soap (Largo®) and tap water. Then after, they were left under running tap water for 60 minutes to reduce load of contaminants on their surface, as well as to expose the hidden microbes within perforations of the explants. Subsequently, the nodal explants were reduced to two nodes each with hanging internodes at both sides and soaked in a solution of 3g 1^{-1} Kocide-101 for 30min. After 3 - 5 times rinsing with sterilized distilled water, the explants were transferred to the laminar air flow hood and

dipped in a 70% ethanol solution for one minute, prior to their exposure to the different sterilization treatments. In the course of sterilization, the explants were subjected to different treatments having varied concentrations (i.e. active chlorine percentage of 1 %, 3 % or 5 %) of the commonly available surface sterilization chemical, local bleach (*Berekina*®), for 10, 15 or 20 minutes duration under aseptic condition. In this experiment, following the recommendations of McKendrick (2000) and Badoni and Chauhan (2010) a 1 % sodium hypochlorite solution (commercial bleach) with 10 minutes exposure time was used as a control. In all cases, the culture vessels containing the explants with the sterilizing agent were gently shaken.

The experiment was laid in a 3 x 3 factorial treatment combination in CRD, i.e. three levels of chemical concentrations (i.e. 1%, 3% and 5 % active chlorine (v/v)) and three levels of exposure time (10, 15 and 20 minutes) with three replications. To enhance the efficacy of the sterilant chemical, two drops of Tween-20 (as a wetting agent) was added into each treatment. In all cases, the explants were thoroughly rinsed (4 times) with sterilized distilled water in a sterilized beaker to get rid of the chemical residues on the explant surface.

Explant culture

Inside the laminar flow hood, the surface sterilized explants were laid on sterile Petri dishes and further excised with the help of sterile forceps and blade to remove the chlorine affected tissues at both ends. In due time, their sizes were reduced to a single node with internodes at both sides. Then after, the trimmed explants were cultured in 370cm^3 volume Manson jars containing 40ml of the conditioning medium, i.e. a PGR-free MS basal medium. Finally, vanilla nodal explants of approximately 2cm length were inoculated on to the conditioning medium. After culturing, the mouth of each jar was properly closed with its cap and sealed with a strip of parafilm.

The jars were then labelled, indicating the media code, culturing date, and name of the clone before they were randomly placed on the Dixon shelves within the growth room. After three days culture on the conditioning medium, non contaminated and surviving cultures were recorded and transferred to the shoot initiation medium, following the recommendations of Gopi *et al.* (2006) and Zerihun *et al.* (2009), so as to produce

sufficient number of explants for the subsequent multiplication experiment. This particular medium consists of MS basal medium supplemented with 30 g l^{-1} sucrose, and added with 2mg l^{-1} BAP and 0.5mg l^{-1} GA_3. Shoots initiated at this stage were sub-cultured onto a PGR-free medium for at least two weeks prior to their use in subsequent experiments, so as to remove any carry-over effect of the plant growth regulators that were used at the previous culture stages.

Unless stated otherwise, in all cases the culture jars containing the explants were placed randomly in the growth room having an average of 50 - 60 % relative humidity, temperature of 24-26^{0}C and 2500lux of light intensity with 16 hr duration of light (Tan, *et al.*, 2010).

Figure 1. Aseptic culture of vanilla explants for initiation; (A) Vanilla explants used for culture initiation (B) Aseptic culture of vanilla explants in biosafty laminar flow hood cabinet (C) Vanilla explants under initiation for shoot multiplication experiment

3.3.2. Experiment II: The effects of combinations and concentrations of BAP and NAA on growth and development of vanilla shoots *in vitro*

After five weeks of culture on the initiation medium, elongated shoots having 4 - 6 nodes were collected and cut into segments of two nodes. These two node segments of vanilla explants were cultured on the multiplication medium at an inclined (45^0) position to

encourage sprouting of multiple shoots around the nodes (Giridhar and Ravishanker, 2004). In this experiment, MS basal medium supplemented with 30 g l^{-1} sucrose with four levels of BAP (0, 1, 2 and 3 mg l^{-1}) in combination with four levels of NAA (0.0, 0.5, 1.0 and 2.0 mg l^{-1}).The P^H of the medium was adjusted to 5.75 before it autoclaved and gelled with 1% agar. Therefore, the experiment was laid in a 4 x 4 factorial combination in CRD, whereby the two factors being BAP and NAA each at 4 treatment levels with three replications and six explants per treatment. 25% L-cystine and 100g myo-inosetol were added as additives. Shoots multiplied at this stage were sub-cultured onto a PGR-free medium for at least two weeks prior to their use in subsequent experiments, so as to remove any carry-over effect of the plant growth regulators that were used at the previous culture stages.

3.3.3. Experiment III: Effect of different concentrations of IAA for best *in vitro* root initiation of vanilla shoots on different strengths of MS basal medium

In vitro grown shoots of vanilla having an average length of 4 cm were used as explants in this rooting experiment. Here, three different strengths of MS basal media (full MS, ½-MS and ¼-MS) supplemented with four levels of Indole-3-Acetic Acid (IAA) (0, 0.5, 1 and 1.5 mg l^{-1}). The medium was added with 20 g l^{-1} sucrose as a carbon source and 2 mg l^{-1} of activated charcoal to darken the medium and to enhance rooting as well. 25% L-cystine and 100g myo-inosetol were added as additives .The P^H of the solution was adjusted to 5.75 before it autoclaved and gelled with 1% agar. The experiment was laid in a 3 x 4 factorial treatment combination in CRD with 3 replications.

3.3.4. Acclimatization

In this study, acclimatization of the vanilla plantlets was carried out following the recommendations of Zerihun *et al*. (2009), who attained 85% success. Accordingly, after initiating roots *in vitro*, the vanilla plantlets were extracted from the culture jars, washed thoroughly with lukewarm water to remove the nutrient medium and solidifying agar from the root surface. Subsequently, the plantlets were planted in a polytube pots filled with sterilized potting mix of well decomposed coffee husk, forest soil and sand at a 1:1:1 ratio, respectively. The plantlets were then kept for two weeks under a plastic tunnel covered with 70 % shade net to ensure provision of high relative humidity (80 – 90 %) and

temperature (24 - 26^{0}C), which was essential for their primary acclimatization. After the stated period of time, the high RH level was gradually reduced to the ambient (60 - 70%) by removing plastic tunnel cover. Later, the plantlets were transferred to a 70 % shade net without plastic tunnel for another two weeks for secondary acclimatization and hardening-off. Finally, they were transferred to a 50 % shade house prior to their field transplanting, as vanilla will grow comfortably under partial shade in its natural agroecology.

3.4. Data Collected

Varied pertinent data were collected from the three experiments in the course of the study. These include:

Percentage of infected plants: is a quantitative character evaluated after the sterilization treatment, so as to determine the effectiveness of the sterilizing chemical concentrations at various durations of explant exposure. These were obtained by counting number of cultures contaminated divided by the total number of explants treated with the same treatment, and multiplied by 100 after three weeks of culture.

Types of infection: is a qualitative character considered in the sterilization experiment. It was obtained by counting the type of contaminants divided by total contaminated explants and multiplied by 100.

Percentage of dead explants: is a quantitative character considered in the sterilization experiment. This data shows the effectiveness of the sterilizing chemical and duration of exposure without being lethal. It was obtained through counting the dead explants due to over concentration or over exposure of the plantlets to the sterilant chemical divided by the total number of cultured explants in the same treatment, and multiplied by 100 after three weeks of culture.

Number of green shoot pieces: is a quantitative character considered in shoot multiplication experiment. The data were obtained through counting normal or actively growing shoots from a single explant after five weeks of culture.

Average length of shoots: is a quantitative character considered in shoot multiplication experiment. The data were obtained through measuring the length of shoots in cm from the stem base to the shoot tip after five weeks of culture.

Number of leaves: is a quantitative character considered in shoot multiplication experiment and data were obtained through counting the number of leaves developed from a single explants and divided by the number of shoots produced from the explant within five weeks of culture.

Number of nodes: is a quantitative character considered in shoot multiplication experiment and data were obtained through counting the number of nodes developed from a single explant and divided by the number of shoots produced from the explant within five weeks of culture.

Fresh weight of shoots: is a quantitative character considered in shoot multiplication experiment by weighing the fresh shoots after five weeks of culture. Upon removal from the medium, the vanilla shoots were washed with in sterile water, dried with filter paper and their weights were measured using a digital sensitive balance and the average fresh weight was considered.

Dry weight of shoots: is a quantitative character considered in shoot multiplication experiment and data were obtained by weighing the shoots using a digital sensitive balance after drying the plantlets micro-shoots in an oven at a temperature of 65°C - 70°C for 48 hrs and the dry weight was considered.

Number of main roots: is a quantitative character considered during the *in vitro* root development experiment. Data were obtained by counting the roots developed from each plantlet after four weeks of culture and divided to the number of plantlets.

Average length of main roots: is a quantitative character considered in root development experiment under the *in vitro* condition, and data were obtained by summing up the lengths of main roots on the plantlets arising from each explant and divided by the number of roots found per explant after four weeks of culture.

Fresh weight of roots: is a quantitative character considered in rooting experiment by weighing the roots after four weeks of culture. Upon removal from the medium, the vanilla roots were washed in distilled water, dried with filter paper and their weights were determined using a digital sensitive balance and the average fresh weight was considered.

Dry weight of roots: is a quantitative character considered in rooting experiment and data were obtained by weighing the roots in a digital sensitive balance after drying the explants in an oven at a temperature of 70°C for 48 hrs and the average dry weight was considered.

Survival percentage: is a quantitative character obtained from the acclimatization trial. The data were obtained by counting the number of normal seedlings developed from a polytube pots after a period of 2 and 4 weeks divided by the number of initial plantlets transferred to the *ex vitro* condition at each of the two stages, and multiplied by 100.

3.5. Statistical Analysis

The collected data were analyzed using the SAS statistical software (Version 9.2) and mean separation were made following the procedure of REGWQ (*Ryan-Einot-Gabriel-Welsch Multiple Range Test*). Linear correlation was applied for steriliant and contamination rate and for shoot parameters. The percentage data of sterilization experiment was transformed using arc sign percentage transformation method (Gomez and Gomez, 1976).

4. RESULTS AND DISCUSSION

4.1. The Effect of Sterilant Chemical Concentrations and Exposure Time on Disinfection of Vanilla Explants

In this study, the differences in the rate of explant contamination due to the combined effects of both concentrations of the sterilizing agent and durations of exposure to the sterilant chemicals were highly significant ($p < 0.01$) (Table 1). Complete loss of cultured explants (100%) due to microbial contamination was recorded when vanilla explants were treated for 10 minutes (the shortest duration) with the local bleach (*Berekina*®) solution containing 1 % active chlorine (Table 2).

Table 1. Mean square values for the effect of different concentrations of sterilizing chemicals (*Berekina*®) and treatment exposure time on contamination rates of vanilla explants at JARC (2010)

Source of variation	Mean squares	
	df	Cont.
Chem.conc	3	3095.05***
Exposure time	2	741.21***
Chem.conc* exposure time	4	29.51**

* = Data transformed using arc sin transformation prior to analysis

Chem.conc = Chemical concentration, df = Degree of Freedom, Cont. =Contamination, *** = $P < 0.001$, ** = $P < 0.01$

A general reduction trend in contamination level of vanilla nodal explants (from 100% to 22.23%) was observed with increasing concentrations of the local bleach solution from 1% to 5% active chlorine together with a concomitant increase in exposure time from 10 to 20 minutes (Table 2). In this experiment, the highest survival rate (72.23%) of explants was observed from a treatment that involved a 20 minutes sterilization of nodal explants with 5% active chlorine *Berekina*® solution, followed by a 15 minutes treatment of explants

with a similar level of active chlorine concentration. In all the cases, local bleach (*Berekina*®) did not exert any adverse effect on the explants, thus the surviving explants from the respective treatments were completely green and actively growing on the initiation media. This could be ascribed to the comparatively lower toxicity of the sterilant chemical, local bleach or *Berekina*®.

In the course of this experiment, 5.54% of explant death was recorded from those combinations involving longer exposure time and the highest level of chlorine concentration (5%) in the sterilant chemical (Data not shown). In this study, nodal explants treated with 5% active chlorine concentration for 20mins exposure time showed a better survival rate as compared to those subjected to the positive control (10 minutes treatment with a 1.0% sodium hypochlorite solution or commercial bleach) (Table 2). In the current study, contamination level of vanilla nodal explants revealed highly significant ($p < 0.001$) and inversely associated with the level of active chlorine concentrations within the local bleach (*Berekina*®) by a coefficient of - 0.80 (Appendix Table 1).

In this experiment, fungal and bacterial contaminants were easily detected by the naked eye, as they produced visible symptoms on the culture medium. Therefore, fungal contaminations were characterized by their "fuzzy" appearance of multitude of colors that are associated with their mycelial growths (Phyto Technology Laboratory, PTL, 2008).

In summary, the major source of contamination in the present study was observed to be of fungal origin (95.4%), unlike the relatively rare appearance of those associated with bacteria (4.6%). Fungal contaminants were white and grayish green in color (Fig. 2 A - C), whereas bacterial contaminants produced mucoid structures with patterns on the culture media (Fig.2-D). In all cases, aggressive and overwhelming growth of the microbes was observed on the culture medium, as compared to that of the explants, whenever the particular treatments involved lower rates of active chlorine concentrations and/or shorter exposure durations of the explants to the sterilant chemicals, and vice versa.

Table 2. Effect of sterilant chemical concentrations and durations of treatment on the contamination rates of vanilla nodal explants after 3 weeks of *in vitro* culture

Treatment	% active chlorine concentration (v/v)	Exposure time (minutes)	*Level of contamination (%)
Control (Na-hypochlorite)	1.0	10	38.90 ± 1.4^{f}
Local bleach (*Berekina*®) solution	1.0	10	100.00 ± 1.4^{a}
	1.0	15	91.70 ± 1.4^{ab}
	1.0	20	88.90 ± 1.4^{b}
	3.0	10	75.00 ± 1.4^{c}
	3.0	15	63.90 ± 1.4^{d}
	3.0	20	44.46 ± 1.4^{ef}
	5.0	10	50.00 ± 1.4^{e}
	5.0	15	38.90 ± 1.4^{f}
	5.0	20	22.23 ± 1.4^{g}
	CV (%)		4.6

*= Data transformed using arc sign transformation prior to analysis

Treatment means within a column followed by the same letter are not significantly different from each other using REGWQ at $P < 0.05$

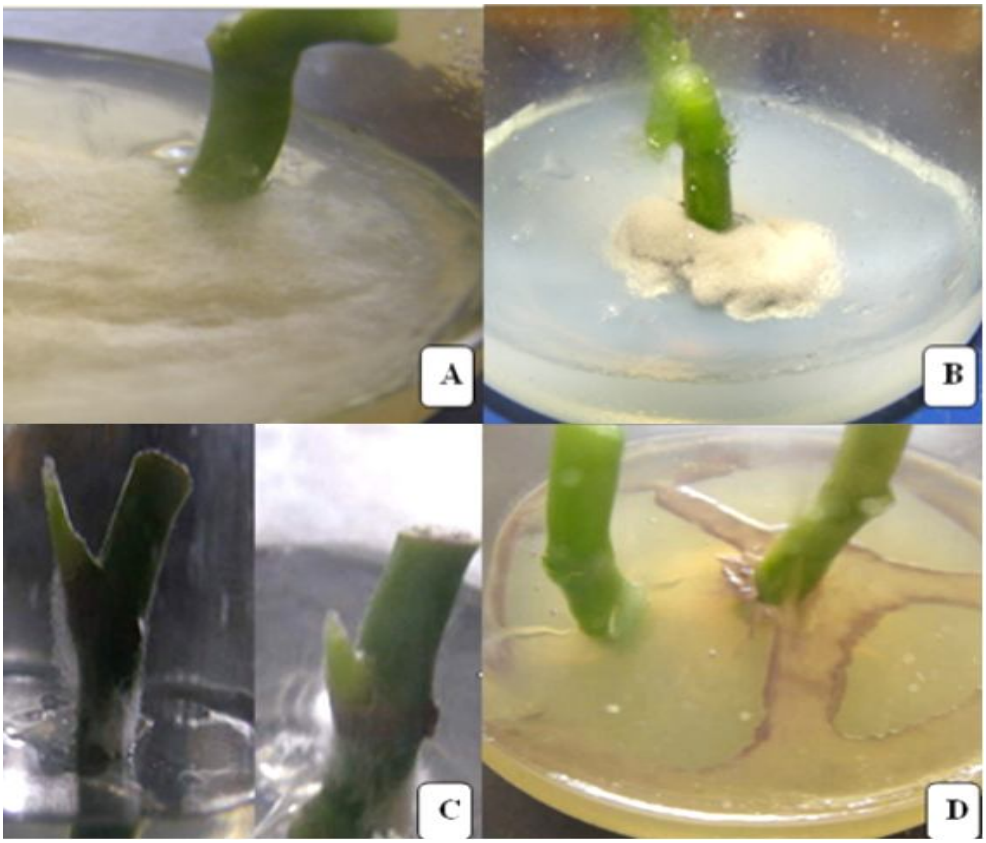

Figure 2. Contamination types observed on *in vitro* culture initiation of vanilla explants; (A), (B) and (C) Different colors of fungal contaminants at different stages of vanilla culture initiation (D) Bacterial contaminants on cultured vanilla explants

The higher prevalence of fungal contamination in those treatments involving lower rates of active chlorine and/or shorter durations of exposure could be attributed to the poor performance of the sterilant chemical at lower concentrations and/or insufficiency of the durations for which the explants were exposed to the chemicals to bring the desired effect. As stated by Shafique *et al.* (2009), it could also be associated with the relatively higher rates of multiplication and mycelial growth of the fungal microflora under the prevailing favorable acidic (pH 5.75) culture medium conditions, which favored more the latter's growth.

Determination of optimal levels of chemical concentrations and durations of explant exposure to the sterilant is highly important to ensure effective control of associated microorganisms and establishment of aseptic culture, without compromising explant

survival. The present study had thus confirmed the possibility of getting up to 72.23% contaminant-free explants, making use of a 20 minutes treatment of nodal pieces of *V. planifolia* using the local bleach *Berekina*® solution containing 5% active chlorine (Table 2).

The efficacy of *Berekina*® on sterilization of vanilla nodal explants in the current study seems a promising alternative to the previous recommendation of Zerihun *et al.* (2009), who only succeeded from using a 5 minutes treatment with 0.1% solution of the hazardous mercuric chloride ($HgCl_2$) solution. The rate of survival in the course of aseptic culture initiation (72.23%) recorded from this study was better than the one obtained by the above mentioned workers (60%). On top of these, the use of *Berekina*® is by far better than that of mercuric chloride, which is strongly discouraged due to its extreme toxicity to plants and animals; beside its costliness, which only make its recommendations so exceptional, i.e. only when other techniques proved ineffective (Parkinson *et al*., 1996). On the other hand, the broad-spectrum efficacy of bleach is particularly ascribed to its peculiar reactivity with microbial cells, i.e. its potentials to react so quickly and denature the cells of most microbial contaminants (Thiel *et al*., 2003). Therefore, use of the locally produced, readily available and cheaper *Berekina*® solution, containing 5% active chlorine, was also preferred to using the commercial sodium hypochlorite (NaOCl) solution, which is an imported product but relatively costly.

4.2. The Effects of Combinations and Concentrations of BAP and NAA on Growth and Development of Vanilla Shoots *In vitro*

In the present study, the combined use of BAP and NAA had substantially promoted several growth parameters associated with the *in vitro* shoot multiplication of vanilla. Therefore, the two-way interaction of these two phyto-hormones had revealed very highly significant differences ($p < 0.001$) with regard to the average number of shoots, leaves and nodes, as well as mean length of the vanilla shoots produced *in vitro* after five weeks of culture (Table 3).

Accordingly, the maximum mean number of shoots proliferated per explant (5.33) and the longest shoots (4.9cm) were recorded from those explants of vanilla cultured on MS basal medium added with 2 mg l^{-1} BAP and 0.5 mg l^{-1} NAA, while the minimum average shoot

number (1.00) and the shortest shoots (1.9cm) were obtained from the culture medium supplemented with 2 mg l^{-1} NAA only (Table 4). However, the average number of shoots recorded from the latter treatment was not statistically different from those supplemented with either 0.5 mg l^{-1} NAA or 1 mg l^{-1} NAA alone

Table 3. Mean square values for the effect of BAP concentrations combined with NAA concentrations on varied shoot growth parameters of vanilla plantlets *in vitro* at JARC (2010)

Source of variation	Mean squares						
	df	Shoot number	Shoot length (cm)	Leaf number	Node number	Shoot fresh wt.(g)	Shoot dry wt.(g)
BAP	3	18.097***	4.974***	1.823***	1.265***	5.484***	2.603***
NAA	3	1.847***	0.892***	0.138***	0.193***	0.212***	0.074***
BAP*NAA	9	1.176***	0.991***	0.153***	0.190***	0.137***	0.076***
Error	32	0.125	0.086	0.049	0.060	0.030	0.016

*** = P < 0.001 BAP= 6-benzyl aminopurine, NAA= α-Naphtalic Acetic Acid

In vitro growing shoots of vanilla had revealed a tendency of callusing when cultured on a medium fortified with sole BAP, at a concentration of 3 mg l^{-1}. On the other hand, the use of NAA alone in the culture medium had negatively affected the average number of shoots and other shoot parameters in the course of vanilla *in vitro* culture. Besides, vanilla explants cultured on a plant growth regulator (PGR)-free MS medium revealed a tendency of root initiation, although they were cultured for shoot multiplication. In addition to this, the shoots produced on this particular medium were not good looking, hence indicating the necessity of using proper types and levels of plant growth regulators to attain proliferation of good quality shoots. In this study, shoot numbers revealed highly significant ($p < 0.001$) and positive correlation with shoot length (0.65) (Table 5).

In the present experiment, the highest mean number of leaves (2.41) and nodes (2.31) per explant were recorded from MS medium added with a combination of 2 mg l^{-1} BAP and 0.5 mg l^{-1} NAA (Table 4). The shoots obtained were thus stout, well elongated and healthy having fully unfolded leaves (Fig. 3 C). However, the numbers of leaves recorded at this level were not statistically different when the culture media were fortified with the same level of BAP (2 mg l^{-1} BAP), but different rates of NAA, i.e. either 1 or 2 mg l^{-1} NAA. On the other hand, the lowest mean number of leaves and nodes (1.0 each) per explant were recorded from the treatment added with 2 mg l^{-1} NAA alone (Table 4).However, the average number of nodes recorded here was not statistically different from those treatments involving sole NAA at a rate of 1 mg l^{-1}.

In addition to these, the correlation between shoot length and leaf number, as well as number of leaves and nodes were observed to be highly significant ($p < 0.001$) and positive, with coefficients of 0.77 and 0.92, respectively (Table 5). The strong positive correlation between the number of leaves and nodes could be ascribed to the inherent anatomy of the vanilla plant, whereby a leaf is always produced at an alternate position on each node.

In the current experiment, supplementing BAP and NAA into the MS basal medium had brought about very highly significant differences ($p < 0.001$) on mean fresh and dry weights of vanilla plantlets derived after five weeks culture *in vitro* (Table 3). Therefore, the highest mean fresh weight of vanilla shoots per explant (2.33g) was recorded from the treatment containing MS basal medium added with 3 mg l^{-1} BAP alone, followed by the one fortified with 2 mg l^{-1} BAP in combination with 0.5 mg l^{-1} NAA (Table 4). However, the medium that gave the highest mean shoot fresh weight didn't result in the highest dry weight accumulation. On the other hand, the highest dry matter accumulation (1.38g) was recorded from the treatment involving 2 mg l^{-1} BAP and 0.5 mg l^{-1} NAA. In the present study, the lowest average fresh weight and shoot dry weight (0.6g) per explant was recorded from MS medium added with NAA (2 mg l^{-1}) alone (Table 4).

Table 4. The interaction effect of different levels of BAP and NAA treatments on *in vitro* shoot multiplication of vanilla after five weeks of culture at JARC (2010)

Treatment			Shoot No	Shoot length (cm)	Leaf no	Node no	Shoot fresh wt.(g)	Shoot dry wt. (g)
No.	BAP	NAA						
T_1	0.0	0.0	2.33^{h}	3.63^{c}	1.78^{e}	1.92^{bcd}	0.85^{j}	0.18^{i}
T_2	0.0	0.5	1.50^{ijk}	2.86^{e}	1.33^{g}	1.55^{e}	0.58^{k}	0.09^{i}
T_3	0.0	1.0	1.33^{jk}	2.43^{f}	1.22^{g}	1.11^{f}	0.38^{l}	0.08^{i}
T_4	0.0	2.0	1.00^{k}	1.90^{g}	1.00^{h}	1.00^{f}	0.35^{l}	0.06^{i}
T_5	1.0	0.0	2.50^{gh}	2.96^{e}	1.93^{de}	1.86^{bcd}	1.04^{i}	0.39^{g}
T_6	1.0	0.5	3.17^{ef}	3.20^{ef}	2.16^{bcd}	1.99^{bc}	1.26^{h}	0.59^{f}
T_7	1.0	1.0	2.00^{hi}	3.50^{cd}	2.00^{cde}	2.05^{ab}	1.16^{hi}	0.65^{f}
T_8	1.0	2.0	1.66^{ij}	3.23^{cde}	1.88^{e}	1.69^{de}	0.87^{j}	0.17^{h}
T_9	2.0	0.0	3.50^{def}	4.50^{b}	2.14^{bcd}	2.09^{ab}	1.58^{g}	0.88^{e}
T_{10}	2.0	0.5	5.33^{a}	4.90^{a}	2.41^{a}	2.31^{a}	2.15^{b}	1.38^{a}
T_{11}	2.0	1.0	3.66^{cde}	4.20^{b}	2.28^{ab}	2.14^{ab}	1.75^{ef}	1.05^{c}
T_{12}	2.0	2.0	4.00^{bc}	3.23^{cde}	2.21^{abc}	2.16^{ab}	1.84^{de}	1.13^{b}
T_{13}	3.0	0.0	3.00^{fg}	2.46^{f}	1.55^{f}	1.50^{e}	2.33^{a}	0.93^{de}
T_{14}	3.0	0.5	4.17^{bc}	3.40^{cd}	2.00^{cde}	1.95^{bcd}	1.93^{cd}	0.89^{e}
T_{15}	3.0	1.0	3.83^{bcd}	2.93^{e}	2.13^{cd}	1.91^{bcd}	2.01^{c}	1.05^{c}
T_{16}	3.0	2.0	4.33^{b}	3.40^{cd}	1.79^{e}	1.73^{cde}	1.703^{f}	0.99^{cd}
CV (%)			7.3	4.5	4.6	5.8	3.8	4.4

T = Treatment, CV = Coefficient of Variation

Treatment means within a column followed by the same letter are not significantly different from each other using REGWQ at $P < 0.05$

Moreover, the correlation between mean shoot length with that of shoot fresh weight and dry weight was found to be highly significant ($p < 0.001$) and positive ($r = 0.46$ and 0.59, respectively). A very strong correlation was recorded between shoot fresh weight and shoot dry weight $r = 0.94$. In addition, leaf number had also revealed highly significant ($p < 0.001$) positive correlation with those of shoot fresh and dry weights ($r = 0.67$ and 0.74, respective) (Table 5).

Table 5. Correlation between different growth and development parameters of vanilla during *in vitro* shoot multiplication at JARC (2010)

Response parameters*	SNO	SL	LNO	NNO	SFW	SDW
SNO	1.00					
SL	0.65***	1.00				
LNO	0.76***	0.77***	1.00			
NNO	0.72***	0.82***	0.92***	1.00		
SFW	0.87***	0.46**	0.67***	0.61***	1.00	
SDW	0.92***	0.59***	0.74***	0.68***	0.94***	1.00

SNO = Shoot Number, SL= Shoot Length, LNO = Leaf Number, NNO = Node Number, SFW = Shoot Fresh Weight, SDW = Shoot Dry Weight

*** = $P < 0.001$ ** = $P<0.01$

The enhanced shoot proliferation recorded in the present study from the combined use of BAP and NAA was in agreement with the reports of several authors (George and Ravishankar, 1997; Gopi, 2006; Zerihun *et al*., 2009). In all these cases, the beneficial effects of using BAP in combination with either NAA or other cytokinins to attain shoot proliferation in the course of vanilla *in vitro* culture had been well established.

The result obtained in this investigation for shoot multiplication and elongation of *V. planifolia* is highly comparable with the previous works done by Giridhar *et al.* (2001) in India. Moreover, the present finding is also in agreement with the study conducted in Indonesia by Hapsoro and Yusnita (1997), whereby apical meristems were used as explant sources. Besides, it was also in accordance with the reports of George and Ravishankar

(1997), as well as Zerihun *et al.* (2009) who exclusively used nodal segments as a source of explants in micropropagating vanilla.

However the present finding is in contrast with the findings of Janarthanam and Seshadri (2008) who used indirect organogenesis from leaf using MS medium supplemented with 1 mg l^{-1} 2,4 D and 0.5 mg l^{-1} BAP for callusing and 3 mg l^{-1} BAP and 2.5 mg l^{-1} NAA for subsequent shoot regeneration with highest average(14 shoots per explant) shoot number. This may be ascribed due to the utilization of different steps and methods of shoot regeneration.

As exemplified in this study, in cases when the concentration of BAP exceeds the optimal level (i.e. 2 mg l^{-1}) with devoid of NAA, the cultures will be liable to produce calli that would obstruct subsequent shoot multiplication. Besides, the use of sole NAA in the shoot multiplication medium would also affect vanilla shoot development negatively, since NAA alone doesn't initiate shoot development. In most of the cases, the combined use of BAP and NAA had proved to be the best in enhancing shoot multiplication, beside its benefits in giving rise to superior responses in most of the shoot parameters considered in this experiment (Table 4). From their micropropagation experiment on *Annona glabra*, Oliveira *et al.* (2008) had also substantiated the benefits of using BAP in the culture medium, since it promotes accumulation of dry matter in the regenerating plantlets.

Plant hormones do not function in isolation within the plant body; instead they also react with each other, as well; consequently, the balance of hormones is apparently more important in the course of any plant growth and development than the absolute concentration of any one hormone. In other words, the balance between cytokinin and auxin plays a detrimental role in the overall growth of plant tissues (Che *et al.*, 2002).Hence the hormone differentials within each medium composition could have different effects on the growth and development of the cultured explants (Mineo, 1990).

The present result is much better than the protocol reported by Giridhar *et al.*(2003), which stated MS medium added with BAP (5 mg l^{-1}) and PAA (1mg l^{-1}) to be the best giving rise to an average of 2 - 3 shoots with 2.32cm length from nodal explants within one month time. This could be associated to the weak chemical nature of the auxin used in their study (PAA) to exert a synergistic effect on vanilla shoot proliferation (George *et al.*,

2007). On the other hand, it could also be ascribed to the relatively higher concentration of BAP used in their case that is thought to be more than enough for enhancing shoot proliferation, which instead might have played an inhibitory role. Moreover, the observed differences in response could also be associated with the differences in the prevailing physiological status of the explants cultured.

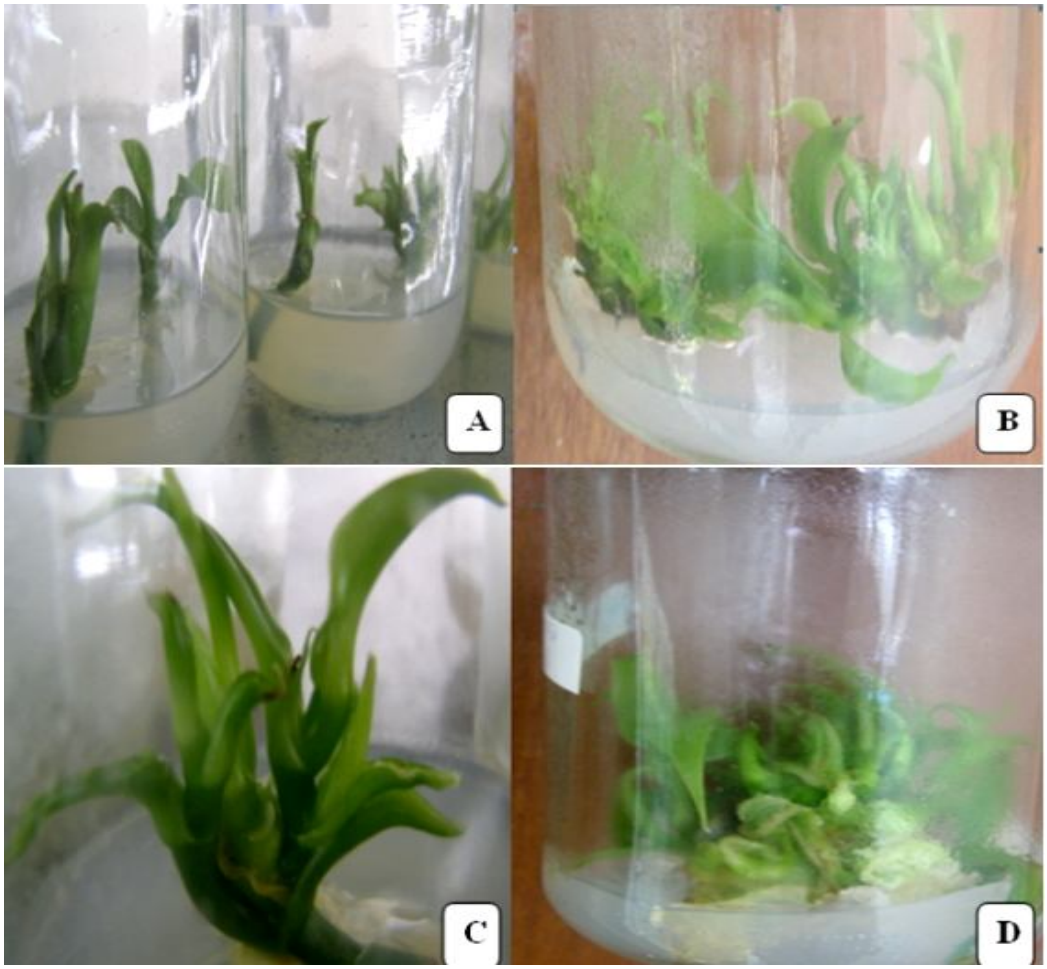

Figure 3. Vanilla micro-shoots at different ages on multiplication media; (A) Vanilla explants a week after subcultured from initiation to multiplication medium, (B) Vanilla plantlets after 3 weeks of cultured on shoot multiplication medium (C) Elongated shoots of vanilla at their fourth week of culture on a multiplication medium (D) Vanilla explants showing callus initiation, i.e. from the treatment involving MS medium added with 3 mg l^{-1} BAP

4.3. The Effect of Different Concentrations of IAA and MS Basal Medium Strengths on the *In vitro* Root Initiation of Vanilla Shoots

In the course of *in vitro* rooting of vanilla plantlets, 100 % root initiation was attained within 7 days of culture on the different rooting media types evaluated in this study. The ANOVA test for the average number of initiated roots showed very highly significant differences ($p < 0.001$) among treatment combinations. This could be attributed to the differential combination effects of the auxin, Indol - 3 -Acetic Acid (IAA), and the MS salt strengths evaluated in the present study (Table 6).

Consequently, the treatment combination containing ½ MS added with 0.5 mg l^{-1} IAA gave the highest average number of roots (4.00) per explant, followed by this same MS salt strength supplemented with IAA at 1 mg l^{-1}(Table 7). However, the later had not revealed any significant difference with the treatments involving a combination of IAA at 1mg l^{-1} with either ¼ or full strength MS basal medium. The average number of roots recorded in this experiment ranged from 1.50 to 4.00 per explant. In general, the medium with ¼ MS strength gave plantlets having roots that are so thin and fragile at any level of the IAA supplement. However, all the rest treatment combinations involving either full or ½ MS salt strengths developed thicker and good looking roots, irrespective of the level of IAA supplement. Meanwhile, the lowest rate of rooting was recorded from those treatments containing either full or ¼ MS basal salt strengths, but devoid of any plant growth regulator supplement (Table 7).

Table 6. Mean square values for the effect of different concentrations of IAA & MS basal medium strengths on the *in vitro* root parameters of vanilla after four weeks of culture at JARC (2010)

Source of variations	df	Mean square			
		Root number	Root length	Root fresh wt.(g)	Root dry wt.(g)
MS strength	2	1.715***	2.05***	0.144***	0.002***
IAA	3	5.675***	4.931***	0.117***	0.001***
MS S * IAA	6	0.280***	1.229***	0.020***	0.0005***

MS = Murashige and Skoog (1962) Medium Strength, IAA = Indiol-3- Acetic Acid
*** = $P < 0.001$

Table 7. The interaction effect of different levels of IAA and MS salt strengths on the *in vitro* rooting of vanilla plantlets after four weeks of culture at JARC (2010)

Treatments			Root number	Root length (cm)	RFW(g)	RDW(g)
No	MS strength	IAA (mg/l)				
T_1	MS	0.0	1.50±0.11^{e}	3.80± 0.09^{h}	0.33± 0.01^{h}	0.033±0.001^{f}
T_2	MS	0.5	2.50±0.11^{c}	5.30± 0.09^{b}	0.44± 0.01^{d}	0.039±0.001^{e}
T_3	MS	1.0	3.16±0.11^{b}	3.40± 0.09^{i}	0.63± 0.01^{b}	0.055±0.001^{c}
T_4	MS	1.5	1.83±0.11^{e}	6.20±0.09^{a}	0.44± 0.01^{d}	0.038±0.001^{e}
T_5	½ MS	0.0	2.00±0.11^{de}	4.40± 0.09^{ef}	0.36± 0.01^{fg}	0.033±0.001^{f}
T_6	½ MS	0.5	4.00± 0.11^{a}	6.10±0.09^{a}	0.76± 0.01^{a}	0.09±0.001^{a}
T_7	½ MS	1.0	3.50±0.11 ^{b}	4.70±0.09^{de}	0.65± 0.01^{b}	0.059±0.001^{b}
T_8	½ MS	1.5	2.33±0.11^{cd}	5.03±0.09^{bc}	0.47± 0.01^{c}	0.043±0.001^{d}
T_9	¼ MS	0.0	1.50±0.11^{e}	3.86±0.09 ^{gh}	0.22± 0.01^{i}	0.021±0.001^{g}
T_{10}	¼ MS	0.5	2.66±0.11^{c}	4.23± 0.09^{fg}	0.37± 0.01^{f}	0.03±0.001^{f}
T_{11}	¼ MS	1.0	3.33±0.11^{b}	3.93±0.09^{gh}	0.41± 0.01^{e}	0.038±0.001^{e}
T_{12}	¼ MS	1.5	2.00±0.11^{de}	4.83±0.09^{cd}	0.34±0.01^{gh}	0.03±0.001 ^{f}
	CV (%)		7.37	3.28	2.3	2.9

* MS = Murashige and Skoog (1962) Medium, T = Treatment, IAA = Indiol-3- Acetic Acid, Acid, RFW =Root Fresh Weight, RDW= Root Dry Weight, CV=Coefficient of Variation Treatment means within a column followed by the same letter are not significantly different from each other using REGWQ at P< 0.05

Likewise, root length had also revealed very highly significant differences ($p < 0.001$) in the present study. In line with this, the longest average roots per plantlet (6.20cm) were recorded from MS medium at full strength supplemented with IAA at 1.5 mg l^{-1} concentration. However, it has no any significant difference with the treatment involving ½ strength MS salts supplemented with 0.5 mg l^{-1} IAA. On the other hand, the shortest roots were recorded from those plantlets cultured on full strength MS salt medium added with 1 mg l^{-1} IAA (Table 7).

Besides, the different treatment combinations had also exerted very highly significant variations ($p < 0.001$) on both fresh and dry weight of the roots (Table 7). Hence, the highest mean root fresh and dry weights per plantlet were recorded from the treatment involving ½ strength MS medium added with 0.5 mg l^{-1} IAA, followed by MS medium of a similar salt strength supplemented with 1 mg l^{-1} IAA. On the contrary, the lowest means for root fresh and dry weights were recorded from the treatment containing ¼ MS salt strength, but devoid of any plant growth regulator (Table 7).

Along all levels of MS salt strengths evaluated in the present study, culture medium amended with the particular auxin used in this study (IAA) resulted in a better root development within four weeks of culture, unlike those treatments devoid of it. In this study, the use of sole IAA had given rise to the formation of more roots. In substantiating the present results, other workers (Gopi *et al.,* 2006; Gantait *et al*., 2009) had also reported the remarkable effect of IAA for *in vitro* root growth and development of vanilla plantlets.

In this experiment, a significant increase in the number of *in vitro* initiated roots of vanilla was observed when the MS basal salt strength was retained at half and the concentration of IAA was kept at its minimum (0.5 mg l^{-1}). Reducing the mineral salt concentrations within the MS basal medium to half had also been reported to enhance the root initiation, as well as other associated root growth and development parameters, together with qualities of the roots produced (George and Ravishankar, 1997; Fotopoulos and Sotiropoulos, 2005).

The results obtained in the current study from the use of ½ strength MS salts supplemented with 0.5 mg l^{-1} IAA are in line with the reports of Gopi *et al.* (2006). The present finding is also in agreement with the outcomes publicized by Giridhar *et al.* (2001) from using the Rimler-Shotts (1973), RS, medium. However, the current findings are in contrast to the reports of Giridhar *et al.* (2003) who succeeded from using MS medium supplemented with 2 mg l^{-1} IBA. This could be attributed to the variation in the efficacy different rooting hormones in relation to the specific vanilla genotype, as well as the interaction between genotype and MS salt strength used in each case.

Rooting response of micro-shoots is stated to be controlled by the type and concentration of plant growth regulators used in the medium (Bhojwani and Razdan, 1992). According to Mineo (1990), auxin compounds generally stimulate cell expansion, particularly cell elongation, besides promoting adventitious root development.

In the current investigation, reducing MS salt strength to half accompanied with the use of a lower level of IAA supplement had proved to be superior in enhancing *in vitro* rooting of *V. planifolia*. Similarly, the promotory effect of reducing the salt concentration to ½ MS in the course of rooting of *in vitro* growing plantlets had been stressed by different authors (Baskaran and Jayabalan, 2005; Hossain, 2008), which substantiates the findings from the current study. However, when the MS salt concentration is reduced significantly to the level of a quarter, the quality of the roots initiated was reduced considerably irrespective of the level of IAA supplement. This could be associated with the insufficiency of the medium salt composition to provide the required nutrient supply for the growth and development of quality roots of vanilla *in vitro*. Successful root initiation in the course of *in vitro* culture is so essential, since it largely determines subsequent *ex vitro* acclimatization and field survival of tissue culture derived plantlets.

4.4. Acclimatization of *In vitro* Derived Vanilla Plantlets

In this study, successful acclimatization of tissue culture derived *V. planifolia* plantlets of with pre-initiated *in vitro* roots was attained after completing all the *ex vitro* associated steps (Fig.4). Consequently, the plantlets had recorded a mean survival rate of 83.4%. In the course of this, *in vitro* derived vanilla plantlets having broader and well developed leaves were the best in their *ex vitro* survival than those with reduced and under developed leaves (Fig. 4).

In the present study, a step wise acclimatization of vanilla *in vitro* derived plantlets helped to overcome the *ex vivo* condition and hence showed good survival rate in the green house and external condition as well (Fig. 4 C-E). Since the plantlets got special care to adapt the external environment and could develop functional stomata and leaf cuticle, thereby start to photosynthesize (Seelye *et al*., 2003). After one month of acclimatization the vanilla plantlets transferred into single polytube pot soil mix, so as to ease transportation of seedlings to its ideal agroecological area in Ethiopia (Fig.4, E).

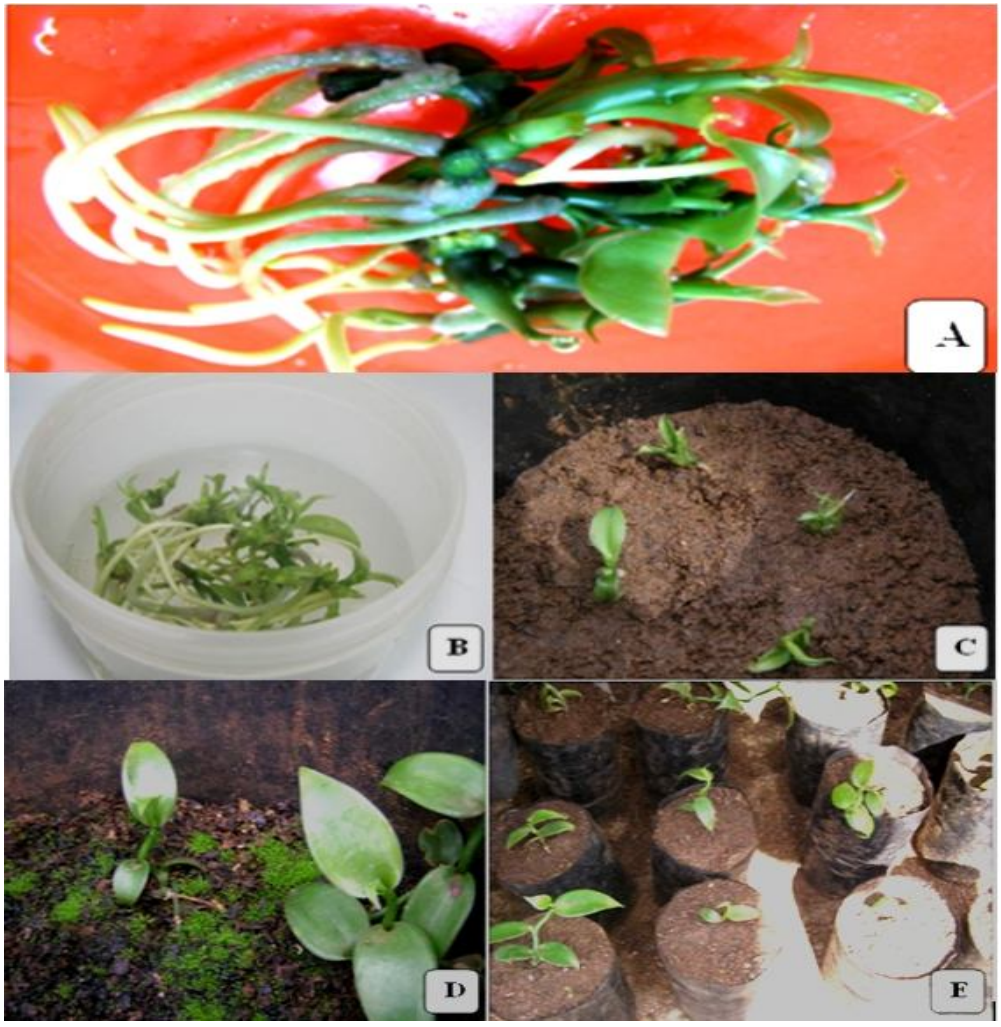

Figure 4. Acclimatization of *in vitro* raised vanilla plantlets; (A) Vanilla plantlets extracted from the rooting medium (B) Vanilla rooted plantlets being washed with lukewarm water, prior to fungicidal treatment (C) Vanilla *in vitro* derived plantlets planted on appropriate soil mix, four plantlets in each polytube pot (D) Survived vanilla plantlets from hardening stresses (E) Survived vanilla plantlets with in single polytube pot after one month of hardening

5. Summary and Conclusion

Vanilla is a commercially cultivated crop worldwide for its fragrant pods, from which the world's most popular flavoring substance, vanillin, is extracted. Although it had been around two decades since the first introduction of vanilla to Ethiopia, it was only in 2005 that the Ethiopian research system had acquired a commercial vanilla clone. Adaptation studies conducted so far had proved its suitability to the hot and humid lowland agroecologies of Tepi and Bebeka. Since this particular clone is represented with only few plants at the Tepi Research Center, the research system is still unable to respond to the prevailing huge demands of vanilla planting material. Particularly, when one considers the limited number of this newly introduced commercial clone, its supply to the users seems farfetched making use of the conventional vegetative propagation. The use of stem cuttings for large scale production and dissemination of planting materials is less efficient, very slow, labor intensive and hence costly.

On the other hand, production of vanilla via tissue culture is stated as the best alternative to conventional propagation. Above all, mass production of clean planting materials of a desired clone is the major concern of vanilla producers all over the world, as well as in Ethiopia. Therefore, it is possible to produce large number of plants throughout the year under controlled growth conditions in a small space through the *in vitro* technique.

In the course of micropropagation, it is essential to make use of all the steps that are indispensable to mass propagate *V. planifolia*. The first of these is sterilization of selected explants, which is a key step for removing any sorts of microbial contaminants from the explant surface; thereby attain successful *in vitro* establishment that is the basis to subsequent growth and development of the tissues. In the course of this study, a 20 minutes treatment of the nodal explants with a 5% active chlorine solution of locally available bleach, *Berekina*®, had successfully cleansed the explants resulting in 72.23% contaminant-free explants established *in vitro*.

The combined effects of different concentrations of BAP, as a source of cytokinin, together with several concentrations of NAA, as a source of auxin, were evaluated for enhanced vanilla shoot multiplication. Consequently, the use of MS basal medium added

with 2 mg l^{-1} BAP and 0.5 mg l^{-1} NAA was found to be the best giving rise to 5.33 shoots per explant that have 4.9cm length on the average after five weeks of culture.

Besides, the effects of three different levels of MS salt strengths were tested in combination with different levels of IAA on rooting of *in vitro* derived vanilla plantlets. Therefore, the use of ½ strength MS medium supplemented with 0.5 mg l^{-1} IAA gave an average of 4.00 roots per micro-shoot that are 6.10cm in length after four weeks of culture. Finally, the rooted vanilla plantlets were extracted from the jars and were subjected to *ex vitro* acclimatization and hardening-off using the recommended 1:1:1 soil mix of forest soil: sand and well decomposed coffee husk, resulting in an average field survival rate of 83.4 %.

The present study is of significant importance in developing a rapid method for multiple shoot proliferation along with efficient root development, and successful plant development, as well. To this end, the availability of this protocol is so pertinent to step up vanilla production in Ethiopia. In this research, an efficient protocol for the micropropagation of a commercial vanilla genotype (Van. 2/05) was developed.

- The sterilization technique developed in the present study would be so beneficial to the spice sub-sector, since it had come up with a suitable technique for effective establishment of vanilla nodal explants under *in vitro* condition. The present finding could also be of paramount importance as it avoids the need for using the imported, hazardous and costly mercuric chloride solution for cleansing explants. Instead, it had devised effective ways for using the locally available bleach, *Berekina*®, at a rate of 5% active chlorine for 20 minutes duration.

- Besides, the present finding had also availed pertinent information regarding the media components required to attain high rate of shoot proliferation of the vanilla accession at hand, (Van.2/05), which was introduced from Mauritius. Culturing nodal explants on MS medium fortified with 2 mg l^{-1} BAP together with 0.5 mg l^{-1} NAA, was identified to be highly effective in producing well elongated shoots within five weeks of culture. The medium composition was also found to be good in providing the best results regarding other parameters associated with shoot growth and development. Therefore, the particular culture medium identified in the

present study was also good in producing strong, healthy, well developed and true-to-type vanilla shoots.

- Likewise, the use of ½ MS basal salt medium added with 0.5 mg l^{-1} IAA were also proved to be so effective for the *in vitro* initiation, as well as subsequent growth and development of roots of this particular vanilla clone. The medium had also proved to be ideal for producing well grown and developed vanilla plantlets having numerous and well developed roots.

- At the final stage, the *in vitro* rooted plantlets of this vanilla clone had also been subjected to acclimatization and hardening-off using all required care. Therefore, the *in vitro* derived vanilla plantlets were successfully acclimatized and hardened-off to give rise to 83.4 % field survival success, making use of a 1:1:1 potting mix of forest soil: sand: and well decomposed coffee husk.

Due to the long duration of this type of experiments and the limited time frame of the research grant, the study couldn't proceed any further. However, this work had already been up taken by the researchers at JARC, where subsequent studies should target production of large numbers of plantlets making use of the current results. To this end, it is imperative to undertake the following studies, so as to come up with a comprehensive micropropagation protocol that enables production and dissemination of millions of plantlets of this clone. To attain this goal successfully, it is essential to consider the following:

- Studies targeting determination of the most ideal sub culturing stage at which one can obtain the highest and optimal rate of shoot proliferation, as well as the optimum sub culturing cycles one should use, without jeopardizing true-to-typeness of the plantlets and also quality of the plantlets produced.

- Applying the protocol developed to address the current development needs with regard to expansion of vanilla plantations in the country, thereby responding to the overwhelming demands of the producers.

Future line of work should proceed to:

- One advantage of micropropagation is production of virus free plantlets so; in the future studies on virus indexing should also be given attention and incorporated.

- Even if the chance to develop such somaclonal variation in small number of cultures, low level of growth hormones and also involvement of direct organogenesis is so small, the genetic stability of the *in vitro* regenerated vanilla plantlets should be investigated and screened under green house condition phenotypically.

- Cryopreservation studies have to be conducted using the protocol developed in this study.

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7. APPENDICES

Appendix Table 1. Correlation coefficients among chemical concentration, duration of exposure and contamination rate for sterilization experiment of vanilla explants at JARC (2010)

	Cont.	Chem.conc	T
Cont.	1.00		
Chem.conc.	-0.79***	1.00	
T	-0.30^{ns}	0.07^{ns}	1.00

Cont. = contamination, Chem.conc = Concentration, T = Time,

*** = P < 0.001 and ns = none significant

Appendix Table 2. MS media composition for *in vitro* culture experiments

Basal media components	**Concentration (mg l^{-1})**	**Stock solution**
Macro nutrients		X 10 (mg/100ml)
NH_4NO_3	1650	16.5
KNO_3	1900	19
$CaCl_2.2H_2O$	440	4.4
$MgSO_4.7H_2O$	370	3.7
KH_2PO_4	170	1.7
Micro nutrients		X20 (mg /200ml)
H_3BO_3	6.2	124
$MnSO_4.4H_2O$	22.3	446
$ZnSO_4.7H_2O$	10.6	212
KI	0.83	16.6
$FeSO_4.7H_2O$	27.8	0.556
Na_2Fe-EDTA	37.3	0.746
*$Na_2MoO_4.2H_2O$	0.25	Concentrate x 1000
*$CuSO_4.5H_2O$	0.025	(g/1000ml) and take
*$CoCl_2.6H_2O$	0.025	10 ml
Organic supplemente		X 20 (mg/200ml)
Myo-inositol	100.0	2000
Nicotinic acid	0.5	10
Pyridoxine -HCl	0.5	10
Thiamine –HCl	0.1	2
Glycine	2.0	40
Sucrose	20000 / 30000	
Agar	10000	