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# *In vitro* Regeneration of Vanilla (*Vanilla planifolia* L.)

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**Abstract**

Vanilla is a high demanding product sold at high prices in world markets. Natural vanilla has high economic value and is theworld's second most expensive spices after saffron. Despite its economic value, it has been one of the least studied species of vanilla plants among other orchid species. Vanilla's synthetic form (vanillin) can be obtained from various chemical methods by using starting materials (coniferin, guaiacol, eugenol and lignin), but the vanilla flavor can naturally be obtained only from vanilla fruit. Micropropagation is an alternative technique for economically important plant production more effectively used with minimum starting material. For this reason, in the study, regeneration of the vanilla plant *in vitro* were carried out. Seed, young leaves, node and aerial parts of *V. planifolia* L. were used as an initial material. The highest callus regeneration (80%) from node explants was obtained in VK4 (MS+1 mg/L 2,4-D + 1 mg/L BAP) nutrient medium, while 100% direct shoot regeneration was provided in VK3 (MS+1 mg/L 2,4-D + 0,5 mg/L BAP) and VK6 (MS+1 mg/L BAP + 0,5 mg/L NAA) nutrient media. In addition, 50% rooting percentage was obtained in VK6 medium. Indirect shoot regeneration ratio was determined as 33,3% in VK2 (MS + 1 mg / L 2,4-D + 0,5 mg / L KIN) and 71,4% of indirect embryo formation was detected in VK4 medium. All embryos stayed in globules stages.

**Keywords:** vanilla, *Vanilla planifolia* L., regeneration, *in vitro*

## INTRODUCTION

Vanilla (*Vanilla planifolia* L.) is a perennial plant species belongs to *Orchidaceae* family [1]. Though the orchids have high commercial value due to the beauty of their flowers, however the economic importance of the Vanilla species is much higher because of using vanillin (4-hydroxy-3-methoxybenzaldehyde) that obtained after curing and drying of the fruits (vanilla beans) to increase the flavor and taste of the beans. Vanilla extract contains about 1.5% vanillin, and it is widely used as antimicrobial agent beside of using in food, medicine, cosmetic and perfumery industries [2].

Vanilla is a very demanding product that is sold at high prices in world markets [1]. It is the second most expensive spice in the world after saffron [3,4, 5, 6]. The natural vanilla obtained by processing the beans of vanilla plant, which has a complex mixture of flavor ingredients. More than 200 volatile aromatic compounds have been identified in its extract. Important components found in vanilla flavor are; vanillin, vanillic acid, vanillyl alcohol, p-hydroxybenzaldehyde, p-hydroxybenzoic acid and p-hydroxybenzyl alcohol [1, 7].

The historical background of Vanilla suggests that the plant is native to Mexico. "Vanilla" comes from the Spanish word "vainilla", which means "little pod" or "black flower". The history of vanilla begins with the Totonaco Indians in ancient Mexico. They were the first keepers of vanilla secrets. In the fifteenth century the Aztecs from Mexico conquered Totonac and soon developed a taste for vanilla beans. Whereas most tribes paid tribute to the Aztecs in the form of maize or gold, the Totonaca sent vanilla beans to the Aztec kings. The Aztecs were defeated by the conquering Spaniard, Hernando Cortez, he returned to Spain with the precious plunder - vanilla beans - which were combined with cacao to make an unusual and pleasing drink. For eighty years, this special beverage was only enjoyed by the nobility and the very rich. In 1602, Queen Elizabeth's pharmacist Hugh Morgan emphasized that vanilla can be used as a flavoring all by itself, and that it can fully uncover in this way in the versatility of exotic beans. Historically, until the middle of the 19th century, Mexico was the chief producer of vanilla. However, in 1819, vanilla beans were shipped by French entrepreneurs to the islands of Rönion and Mauritius in hopes of producing vanilla there (http://vanilla.servolux.nl/vanilla_history.html).

Due to the destruction of the original habitats particularly those of the tropical regions of America, many of them are considered rare or endangered [8, 2]. The Vanilla genus contains approximately 110 species, but 3 species are considered important: *Vanilla planifolia* Andrews (*Vanilla fragrans* Ames); *Vanilla pompona* Schiede and *Vanilla tahitensis* JW Moore. *Vanilla planifolia* is the most grown species because it is valuable in terms of the quality of the aroma. Vanilla (*Vanilla planifolia* L.) is a climber terrestrial orchid suitable for warm, moist tropical climates [5].

**Taxonomy**

**Table 1.** Vanilla Taxonomy [9].

| | |
|---|---|
| **Family** | *Orchidaceae* |
| **Sub-family** | *Orchidoideae* |
| **Section** | *Acrotoneae* |
| **Tribe** | *Neottieae* |
| **Sub-tribe** | *Vanillae* |
| **Genus** | *Vanilla* |

The worldwide increase in the use of herbal products has also led to an increase in natural vanillin demand. The price of vanilla in the international market varies depending on political relations and climate in regions where production is required [5]. Among the countries producing large quantities of natural vanilla in 2007, Madagascar was the first, while Indonesia became the largest producer of vanilla in 2010 [10, 11].

In India, the total area for vanilla cultivation is about 2545 ha [5]. In Brazil, vanilla production is still at an early stage, although it has the appropriate climate and soil condi-

tions for growing the vanilla. Generally, in Brazil, the seedling market almost disappears due to the low yield of conventional clonal propagation techniques [2].

Global synthetic vanillin production is concentrated in France, the United States, Norway, Japan and China. The "Solvay Group" is the world's largest producer of vanillin, and in 2011 it merged with the Rhodia Company of France, with 48-50% of the global vanilla. The Borregaard Company in Norway is the second largest producer of vanilla in the world, and is the only company to produce lignin-derived vanillin market [12].

Since vanilla is an expensive and sought-after spice, it has been studying for many years to develop a natural, effective and reliable vanillin production method. The use of synthetic vanillin in precious food products is restricted due to concerns about safety. For this reason, the market price of high-quality natural vanillin has increased steadily over recent years [13]. Because of the high cost of growing, harvesting and extracting the vanilla bean, most vanilla flavored aroma "essences" sold commercially is synthetic vanilla made from coniferin, guaiacol, eugenol and lignin paste or clove oil. 1,5-3 kg of pods can be produced from a five-year-old vanilla plant and increases in the number of pods obtained each year [5, 12].

As of 2011, annual vanillin production in the world is reported to be approximately 16,500 tons. This production is based on petrochemical sources which is about 15,000 tons (91% of global supply), 500 tons of which are produced from lignin, and only 30 tons are made from natural vanilla beans. The price of petrochemical-derived vanillin varies between $ 12-25 / kg, the price of lignin-derived vanillin is 100-200 $ / kg [12]. However, while the price of natural vanilla extract varies from $ 1,200 to $ 4,000 / kg. In general, the global vanillin market is estimated at $ 400-700 million [14].

Despite its economic value, vanilla is one of the least studied among other orchid species [15]. Vanilla plants are traditionally regenerated by stem cuttings, and this type of propagation leads to damage to the main plant. *In vitro* tissue culture techniques are considered as an alternative production method to prevent this problem [16].

According to a study carried out by Divakaran et al. (2015), vanilla seeds were cultured in MS nutrient medium supplemented with different auxin and cytokinins and examined for germination, growth and reproduction. They studied production of single and multiple shoots. The transformation of root meristem into shoots occurred in MS medium containing 1 mg / L BAP and 0.5 mg / L IBA [8].

Janarthanam and Seshadri (2008), and Tan et al. (2011) used leaf and node explants for callus regeneration. According to Janarthanam and Seshadri (2008), better callus regeneration was achieved in vanilla leaf explants than in node explants. When the explants were cultured in MS medium containing 1.0 mg / L 2,4-D and 0.5 mg / L BA. The callus regeneration rate of node explants was 35%; while it was 60% in young leaf explants. Tan et al. (2011) reported that the callus formation rates from both node and leaf explants were 35% (MS medium supplemented with 2 mg / L NAA and 1 mg / L BA) and 10% (MS medium supplemented with 2 mg / L NAA and 2 mg / L BA) [17, 18].

In another study conducted by Sharma and Bora (2015), an effective callus initiation protocol was developed using young leaves, shoots and node explants for *Vanillia planifolia*. Explants were cultured in MS mediums containing combinations of 2,4-D, NAA and BAP at different concentrations, and callus development was attempted. Nod explants were used as the most successful starting material [19].

Turkey is one of the leading countries of the world in exporting medical and aromatic plants and exports many medical plants while at the same time realizing the extraction of many plants. In addition, Turkey has a great economic potential in terms of medical and aromatic plants collected from nature in terms of having different climatic and ecological conditions and containing many plant species and diversity of floras [20].

Although some regions of Turkey have the appropriate climate to grow vanilla plant, however this plant is not cultivated in Turkey. For this reason, in this work, regeneration of the vanilla plant in *in vitro* conditions were carried out. In the study, vanilla plant's seeds, stem and leaf explants were used to provide callus, shoot and embryo regeneration using nutrient media contained appropriate plant growth regulators.

## MATERIAL and METHOD

### Material

In our study, seeds, young leaves, nods and aerial roots explants of mature vanilla (*Vanilla planifolia* L.) used as starting materials. Vanilla beans supplied from İZMİR-BORNOVA and KEMERALTI traditional medicine shops, while nod, leaf, shoot and aerial root explants obtained from Ege University Botanic Garden and Herbarium Applied and Research Center.

### Method

After sterilization of seeds which were obtained from vanilla beans, they cultured for germination in *in vitro* conditions. Tetrazolium test applied to determine the viability of the seeds. The explants, which were taken from the whole plant, sterilized by different sterilization methods and then transferred to nutrient media containing growth regulators in different compositions to provide callus regeneration. MS (Murashige & Skoog, 1962) used as basic nutrient medium. In addition, MS media containing hydrophilic cotton which are soaked in sterile water tested for seed germination experiments.

### Preparation of Stock Solutions

In order to facilitate the preparation of the MS nutrient medium in the laboratory, stock solutions were prepared by chemically compatible grouping. The prepared stock solutions were calculated according to the principle of "5 ml stock solution for 1 liter of nutrient medium" and nutrient media were prepared considering this rule.

### Preparation of Seed Germenation Media

Some of the hydrophilic cotton placed in the jars to prepare the cotton environments to be used for germination of the seeds. These cottons were soaked in distilled water and manually placed on the bottom of the jars so that the top surface was as flat as possible. After the cotton has been soaked and the water has been completely absorbed, the jar lid tightly sealed and labeled. As distinct of cotton medium, the nutrients media given in Table 2 are used for germination of vanilla seeds as well.

**Table 2.** Seed germination media*

| Basic nutrient media | Plant growth regulator composition |
|---|---|
| MS | - |
| MS | 1mg/L BAP +0,5mg/L NAA |

*Sucrose: 30g/L, Gelrite: 3 g/L, pH: 5,7

**Preparation of Callus Regeneration Media**

MS nutrient media containing different compositions of growth regulators were used for the initiation of callus regeneration from different vanilla explants. The compositions and codes of nutrient media are given in Table 3.

**Table 3.** Media compositions for callus and organ regeneration**

| Media Code | Basic Media | 2,4-D (mg/L) | KIN (mg/L) | BAP (mg/L) | NAA (mg/L) |
|---|---|---|---|---|---|
| VK1 | MS | 1 | 1 | - | - |
| VK2 | MS | 1 | 0,5 | - | - |
| VK3 | MS | 1 | - | 0,5 | - |
| VK4 | MS | 1 | - | 1 | - |
| VK5 | MS | 2 | 0,5 | - | - |
| VK6 | MS | - | - | 1 | 0,5 |

**Sucrose: 30g/L, Gelrite: 3g/L, pH: 5,7

**Preparation Steps**

First of all sterilization of nutrient media was performed by autoclaving at 121 °C for 15 minutes at 1.2 atm pressure and culture vessels containing sterile nutrient media were stored appropriately until use.

**Sterilization of Equipments**

Equipments to be used for laminar flow cabinet work should be sterilized before starting the work. All equipment sterilized at 170 °C for one hour after wrapping with aluminum foil. In addition, distilled water which is used for rinsing in sterilization, autoclaved at 121 °C for 15 minutes.

70% ethyl alcohol and 0.1% HgCl2 solutions were prepared for using in surface sterilization. Since commercially available ethyl alcohol solution is 96%, sufficient 70% ethyl alcohol solution was prepared by adding 26 parts of reverse osmosis water on 70 parts of ethyl alcohol. For preparing 0.1% HgCl2 solution, 1 g powder of HgCl2 transferred to 1L volumetric flask then dissolved by adding distilled water on it and the volume completed to 1 L with distilled water. The HgCl2 solution's preparing process performed under hood and using gloves. Magnetic stirrer also used for better dissolution. The solution stored in a specially marked bottle.

**Sterilization of Vanilla Beans**

Vanilla beans were sterilized in the cabinet. For this purpose, the surfaces of vanilla beans were burned after being immersed in 70% ethyl alcohol, as indicated by Divakaran [8]. Then the pods were taken on sterile tiles and divided longitudinally into two pieces with scalpel, and the seeds were allowed to come out. The seeds were fallen down by stripping with a scalpel on the nutrient media. Culture vessels containing seeds kept in dark for callus regeneration.

**Seed Viability Test (Tetrazolium Test)**

Tetrazolium test was used to determine seed viability according the procedure indicated by Vujanovic [21] (http://www.2020seedlabs.ca/what-tetrazolium-chloride-test). The seeds which is taken from vanilla beans weighed in varying amounts between 3-5 mg and transferred to Eppendorf tubes. A 10% sucrose solution was added to the seeds and allowed to stand at room temperature (24 °C) for 24 hours. After 24 hours, the sucrose solution was removed with a pasteur pipette and 1 ml of 1% Tetrazolium solution was added on the seeds. Eppendorf tubes were kept in a water bath at 40 °C for 24 hours. After sufficient treatment of the seeds with the solution, the dye was removed with a pipette and water was added to the seeds. The seeds were taken on a slide and examined under microscope. Live seeds are observed as normal red color because they are resistant to Tetrazolium binding. Weak seeds exhibit abnormal coloration, while dead seeds discolored.

**Sterilization of Vanilla Explants**

The pre-washing process was applied to the vanilla explants which are taken from whole plant. Sterilization process undergoes the following steps: first a few drops of detergent were put in distilled water and shacked for 10 minutes. Then, it was rinsed under running water for 7 minutes to complete the pre-wash process. Meanwhile, scalpel and tissues papers were placed in laminar air flow cabinet with jars containing 70% ethyl alcohol, 0.1% HgCl2 and sterile distilled water and incubated under UV for 15 minutes to provide surface sterilization.

In the laminar flow cabinet, explants first immersed in 70% ethyl alcohol for one minute followed by immersing in 0.1% HgCl2 for 5 minutes. In order to remove sterilizer agents, explants were rinsed 3 times with sterile distilled water and then cultured on different nutrient media.

**Culture of Vanilla Explants**

The sterilized vanilla explants (leaf, stem and aerial roots) were cut into different sizes and transferred to nutrient media. Thin layer technique (thin cell layer-TCL) was applied to the explants. Transverse and longitudinal thin sections were taken from leaf and stem explants, while cross sections were taken from aerial roots (1-2 mm cross section thickness). In each culture vessel, 8 explants for stems, 10 explants for leaves, and 3 explants for aerial roots were established. Culture vessels were transferred to dark culture conditions in order to provide callus regeneration.

**Subculture of Vanilla Calli**

Subcultures were applied every 50 days on calli obtained from cultured explants. Then the callus regeneration ratios were evaluated. VK6 medium (MS + 1 mg / L BAP + 0.5 mg / L NAA), the most successful nutrient medium composition, was used for callus subcultures. Calli were taken from their old media and transferred to new nutrient media (Figure 1).

**Statistical Evaluation of Data**

According to the randomized parcel trial design, during the study period, the data obtained as three repetitive results were evaluated using the SPSS 16.0 statistical program (SPSS Inc., Chicago, USA).

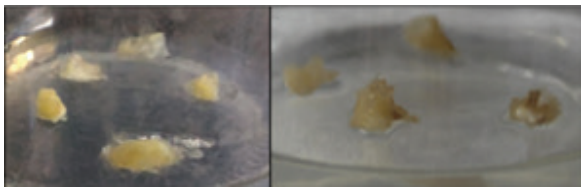

**Figure 1.** Subculture of regenerating callus.

## RESULTS

**Seed Viability Test**

In the result of viability test in which seeds are stained by Tetrazolium chloride, all vanilla seeds were colored. This shows that the seeds are alive.

**Seed Germination Trials**

Seed sterilization shows 100% success, despite of this no germination observed from seeds. However 1mm in diameter light creamy callus like buddings were observed around the seeds. These buddings were mostly expected to show similar features and developed to callus, but this callus did not show any differentiation (Figure 2).

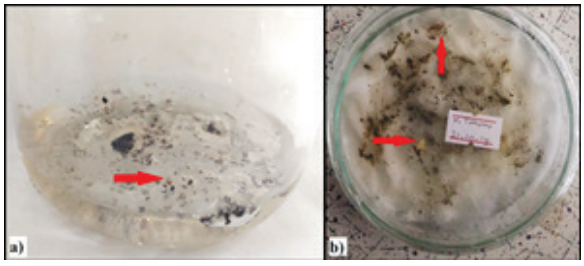

**Figure 2.** Cultured vanilla seeds. a) Vanilla seeds in the nutrient medium. b) Vanilla seeds in cotton medium.

**Callus and Organ Regenerations from Vanilla Explants**

The ratio of callus regeneration was low in cultured vanilla explants. All of the leaf explants showed browning and no improvement was detected. Regeneration of shoots were observed in only 1 cultured node explant. This shoot was then cultured in the same medium, and compacts calli were obtained (Figure 3).

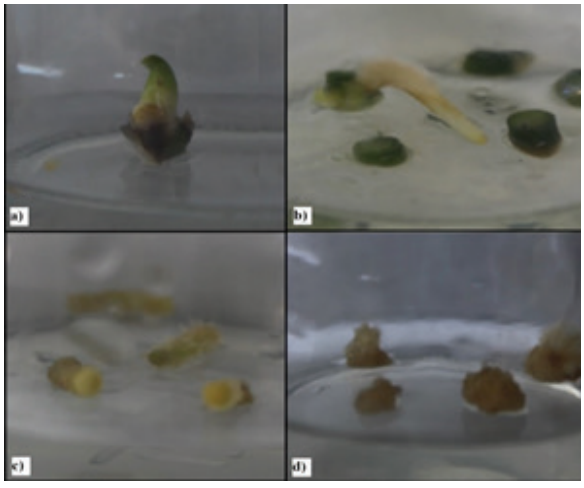

**Figure 3.** Callus and organ regeneration as a result of cultures. a), and b) shoot formation from node explants, c) callus formation from node explants, d) callus formation in subculture.

Regenerated shoots from cultured tissues, showed white color because of dark cultural conditions (Figure 4, a). In order to improve the regeneration, the culture vessel was taken under photoperiod conditions and green vanilla plantlet with aerial roots was obtained. Callus culture is provided from this green plantlet (Figure 4, b and c).

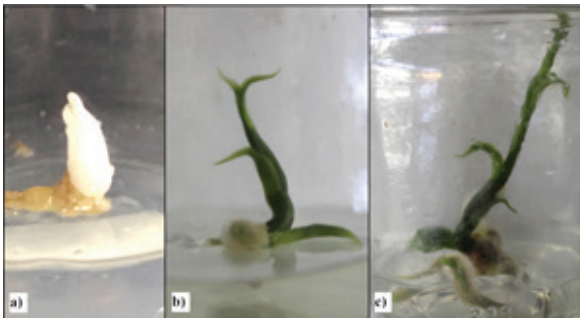

**Figure 4.** Vanilla plantlet development in culture vessel. a) Shoot which is regenerated in dark culture conditions, b) Shoots status after 3 weeks of keeping in photoperiod, c) After 7 weeks

In this study, maximum callus induction (83.3%) was observed from cultured node explants within 35 days in VK4 media. The obtained calli are compact and creamy white in color. In addition, shoot organogenesis and globular embryo formation was observed. No other callus formation or organogenesis was observed in other cultured explants (Table 4).

Shoot formation was seen in all combinations, but the highest percentage of shoot formation (100%) was obtained from VK3 and VK6. The highest percentage of callus formation (83.3%) was observed in VK4 followed by VK1 (50%), VK5 (27.6%) and VK2 (16.7%). However, no callus formation was observed in VK3 and VK6. Root formation (66.7%) observed only in VK6. Other combinations did not show any root regeneration (Table 4).

The highest ratio of globular embryo formation from node explants was observed in VK4 (72.2%) followed by VK1 (33.3%), VK2 (16.7%) and VK3 (11.9%). In VK5 and VK6 no globular embryo formation was observed. The percentage of shoot formation was 8.9% and 6.8% respectively, however the highest percentage of shoot formation obtained from VK4 (38.9%) and the shoot formation in other nutrient media was determined as follow: 33.3% in VK2, 16.7% in VK1 and 9.9 in VK3. When nutrient combinations were evaluated in terms of root formation, successful results were obtained from VK6 (24.4%) and VK3 (12.3%). For all that no root formation was observed in other nutrient media (Table 5, Figure 5). Shoots organogenesis from callus explants shown in Figure 6.

## DISCUSSION/CONCLUSION

Vanilla, with high economic value, have various problems in production. Vanilla plants propagate by stem cuttings rather than seed which causes damages to the main plant. *In vitro* tissue culture techniques are considered as an alternative propagation method to prevent these damages.

In this study; various explants from *Vanilla planifolia*, including seeds and other parts of the plant, were selected and then used in experiments according to different parameters. Explants cultured on MS media containing different growth regulator compositions and kept in dark conditions in order to obtain callus regeneration which is occurred properly in some explants as result of accomplished study. In addition, direct shoot regeneration and root growth have also been observed, making the study exceptional.

**Table 4.** Callus and organ regeneration in node explants cultured in different nutrient media.

| Nutrient Media | Average number of explants cultured (No.) | Average number of callus from node explants (No.) ± SD* | Average Percentage of callus from node explants (%) | Average number of shoots from node explants (No.) ± SD | Average Percentage of shoots from node explants (%) | Average number of roots from node explants (No.) ± SD | Average Percentage of roots from node explants (%) |
|---|---|---|---|---|---|---|---|
| VK1 | 4,0 | 2,0 ± 1,7 | 50,0 | 1,0 ± 1,7 | 33,3 | 0,0 ± 0,0 | 0,0 |
| VK2 | 5,0 | 1,0 ± 1,7 | 16,7 | 1,0 ± 1,7 | 16,7 | 0,0 ± 0,0 | 0,0 |
| VK3 | 2,0 | 0,0 ± 0,0 | 0,0 | 2,0 ± 1,7 | 100,0 | 0,0 ± 0,0 | 0,0 |
| VK4 | 5,0 | 4,0 ± 1,7 | 83,3 | 2,0 ± 1,7 | 33,3 | 0,0 ± 0,0 | 0,0 |
| VK5 | 7,0 | 2,0 ± 1,7 | 27,6 | 2,0 ± 1,7 | 27,6 | 0,0 ± 0,0 | 0,0 |
| VK6 | 4,0 | 0,0 ± 0,0 | 0,0 | 4,0 ± 1,7 | 100,0 | 2,0 ± 1,7 | 66,7 |

*SD: Standard Deviation

**Table 5.** Callus regeneration status.

| Nutrient Media | Average number of callus subcultured (No.) | Average number of embryos from callus explants (No.) ± SD | Average Percentage of embryos from callus explants (%) | Average number of shoots from callus explants (No.) ± SD | Average Percentage of shoots from callus explants (%) | Average number of roots from callus explants (No.) ± SD | Average Percentage of roots from callus explants (%) |
|---|---|---|---|---|---|---|---|
| VK1 | 5,0 | 2,0 ± 1,7 | 33,3 | 1,0 ± 1,7 | 16,7 | 0,0 ± 0,0 | 0,0 |
| VK2 | 2.3 | 1,0 ± 1,7 | 16,7 | 1,0 ± 1,7 | 33,3 | 0,0 ± 0,0 | 0,0 |
| VK3 | 33,0 | 3,0 ± 3,0 | 11,9 | 3,0 ± 0,0 | 9,9 | 4,0 ± 1,7 | 12,3 |
| VK4 | 7,0 | 5,0 ± 1,7 | 72,2 | 3,0 ± 3,0 | 38,9 | 0,0 ± 0,0 | 0,0 |
| VK5 | 31,0 | 0,0 ± 0,0 | 0,0 | 3,0 ± 3,0 | 8,9 | 0,0 ± 0,0 | 0,0 |
| VK6 | 18,0 | 0,0 ± 0,0 | 0,0 | 1,0 ± 1,7 | 6,7 | 4,0 ± 1,7 | 24,4 |

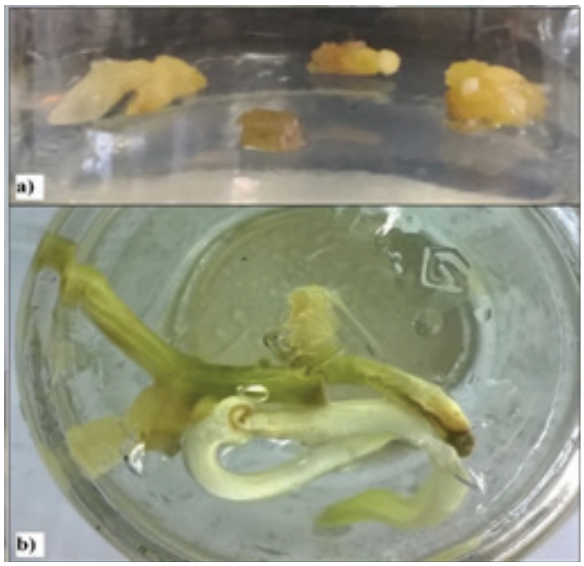


**Figure 5.** Globuler embryos and organ regeneration from callus explants: a) Globuler embryo formation, b) Shoot and root formation.

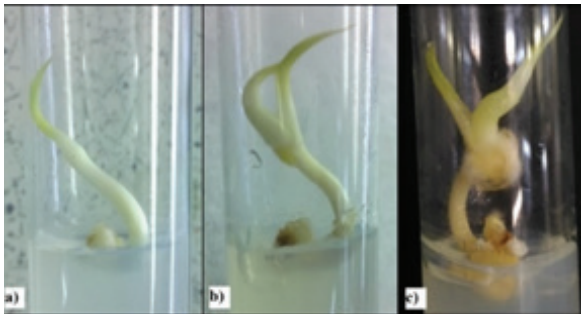


**Figure 6.** Organogenesis of shoots observed in callus explants. a) After 4 weeks of culture, b) After 7 weeks, c) After 11 weeks.

Although the result of seed viability test showed that the seeds are alive and also in previous studies some researcher found that the vanilla seeds are able to germinate in *in vitro* conditions, however in our study seed germination was not observed.

Browning observed in all of the cultured leaf explants and no regeneration was obtained from the leaves. Since HgCl2 used as sterilization agent even at low doses and for a short time it can cause loss of tissue viability due to its toxic properties. In addition, the plant growth regulators which are added to the nutrient media in order to regenerate callus may cause browning and tissue death due to increase of phenolic contents in leaf tissues.

Janarthanam and Seshadri (2008) [17] and Tan et al. (2011) [18] used leaf and node explants in callus cultures. According to the information obtained from the studies, the callus regeneration ratio of node explants is 35% while young leaf explants provide 60% callus regeneration. Tan et al. (2011) [18] reported that the callus formation ratio of node and leaf explants was 35% and 10% respectively. In our study, despite the use of similar media no callus formation detected. Moreover, the effects of two different auxins (2,4-D and BAP) on callus induction were investigated which showed positive results. The results obtained by Sharma and Bora (2015) [19] are similar to those of our study. We found that node explants of *V. planifolia* performed better for callus and organ regeneration.

As a result of our study, VK4 is more effective for callus formation from nodal explants, while VK3 or VK6 is ideal for shoot formation. When evaluated for embryo and organ regeneration from callus, VK4, VK1 and VK2 were more effective respectively. In our experiments, node explants for callus initiation found to be better than leaf explants. In the study, young node explants showed good callus regeneration.

The results obtained from our study shows a higher success rate than the studies carried out so far, and it is the first study to be carried out in Turkey in this respect as well.

## RECOMMENDATIONS

There are many factors that affect the success of *in vitro* micropropagation of different vanilla explants. By evaluating these factors, efforts to achieve more successful results can be carried out. The plants which are used in our study grown in the greenhouse conditions. Literature information has shown that the growth conditions of the donor plant is an important factor in vanilla culture. In addition, explants and tissue types are factors that affecting callus regeneration.

The informations obtained from culturing vanilla seeds on different media lead to perform further researches on seed culture of vanilla by using different new media to achieve appropriate results.

We used dark conditions as culture conditions. Photoperiod or recent interest in the effects of LED on plant regeneration needs to be discussed in the future works.

Our study revealed that vanilla explants showed browning intensively. It may be advisable to change the sterilization method to prevent these declines. Instead of using HgCl2, another sterilization agent such as sodium hypochlorite (NaClO) can be used as well as changing the application period. The addition of active carbon, or agents used in darkening problems, such as ascorbic acid (C6H8O6) and citric acid (C6H8O7) may also offer to the culture medium as alternative ways for solving the problems.

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