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## Effect of methylisothiazolinone biocide in tissue culture sterilization of *Casuarina equisetifolia*

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**Abstract.** In this study, modification of tissue culture sterilization techniques using methylisothiazolinone biocide (MB) in liquid and solid medium was used to evaluate the acquisition of axenic *Casuarina equisetifolia* culture in vitro with nature explant sources. One experimental unit had 12 treatment combinations with 20 replications of explants in each treatment. Acquisition of the best axenic culture of *C. equisetifolia* after 30 days of incubation was in a sterilization combination for 3 days shaking out in liquid WPM medium with 8 mg/l MB and in solid WPM medium with 6 mg/l MB. *C. equisetifolia* shows a good growth response in WPM medium without the addition of exogenous growth hormone. The first indication of axillary shoot explants were elongation response within 15 days of culture initiation. Shoot tips changed in color from dark green to a more obvious transparent green and the internode sections became swollen and barrel-shaped. Single shoot formation from axillary buds at basal nodes and sprouted lateral buds were visible within 30 days. The 90% of shoot formation in the axenic culture of *Casuarina equisetifolia* were from lateral buds.

### 1. Introduction

The worldwide consumption of paper and board products has increased from 125 million tons in 1970 [1] to 402 million tons in 2011, and it is expected to reach 521 million tons per annum by year 2021 [2]. In order to meet future demand and to overcome the wood shortage, studies have been conducted worldwide to evaluate the potential of new or alternative resources as raw material components for pulp and paper production [3]. The use of fast growing species such as *Casuarina equisetifolia* L. Johnson can be a promising alternative of fiber source for paper making.

The *Casuarinaceae* family includes four genera, i.e. *Allocasuarina* L. Johnson with 59 species, *Casuarina* L. Johnson with 17 species, *Ceuthostoma* L. Johnson with 2 species and *Gymnostoma* L. Johnson with 18 species. *C. equisetifolia* is native to the tropical and subtropical coastlines of Australia, Southeast Asia, Malaysia, Melanesia, and Polynesia and New Caledonia [4]. *C. equisetifolia*



is a nitrogen-fixing, evergreen tree 15 to 30 m or more in height and up to 50 cm d.b.h., predominantly a coastal species and has a rare property of growing upright and symmetrical on windswept coasts. On suitable sites, *C. equisetifolia* is one of the world's fastest-growing trees [5]. Seedlings may reach 3 m tall 1 year after planting and 8 m tall and 7 cm d.b.h. after 4 years. At age 15, plantation trees may be 17 m tall and 13 cm d.b.h. In these fast-growing plantations, mean annual increments may reach average 4.5 m<sup>3</sup> per year. The potential of *C. equisetifolia*, systematic tree improvement program and wood fiber characteristics in selected clones are being undertaken in India for over two decades [6]. Pulp and paper making properties of *C. glauca* cooked by ASAM process are observed to be superior to *Casuarina* pulp obtained by the kraft process [7].

Tissue culture techniques, a system of growing explants under aseptic condition, can be an alternative for the continuous provision of plantlet clone stocks for large scale plantation of pulp and paper industry. Although aseptic conditions are usually employed, plant cultures may not stay aseptic *in vitro*. Microbial contamination is a constant problem, which often compromises the development of *in vitro* cultures [8]. Often trees on the forest cannot provide vegetative material (rejuvenation or macro cutting) for explants (cells, tissue or organs that have been separated from mother trees). The problem of contamination is exacerbated when explants are sourced directly from field grown plants [8], even more in tropical forest that have higher diversity of contaminant compared to temperate forests. Contamination is not always visible in the early phase of the culture, and sometimes contamination can also be caused by contaminants from endogenous plants where it is difficult to do the sterilization process. Overexposing tissues to decontaminating chemicals can also kill tissues, so there should be a balancing act between sterilizing explants and killing the explants themselves [10]. 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 axenic cultured tissues *in vitro* [9].

The living materials should not lose their biological activity and only contaminants should be removed during sterilization. The success of sterilization methods is dependent upon the concentration and duration of anti-microbial agents [11]. MB (hereinafter referred as MB) is a relatively new anti-microbial agent, broad-spectrum preservative and biocide for use in plant tissue culture. The active ingredients are 5-chloro-2-methyl-3(2*H*)-isothiazolone and 2-methyl-3(2*H*)-isothiazolone. MB is effective against both bacteria and fungi, is heat stable, and, unlike conventional antibiotics, can be autoclaved in the media [12]. 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 in the end. These microbes compete adversely with plant tissue cultures for nutrients, and their presence often results in increased culture mortality or can also result in variable growth, tissue necrosis, reduced shoot proliferation and reduced rooting [13]. To reduce the level of microbial contamination of the stock plant and hence obtain axenic explants (a culture without foreign or undesired life forms but may include the deliberate co-culture with different types of cells, tissues or organisms), various strategies have been and are still being employed in tissue culture laboratories. According to research conducted by [14] using seedlings nodal segments of *C. bransiliense* from greenhouse, it was shown that use of MB was highly effective in combating bacteria, with the concentration of 0.4% eliminating them after 30 days of culture. MB was evaluated as an alternative to the use of conventional antibiotics and fungicides with leaf explants of chrysanthemum (*Dendranthema grandiflora* Kitam), European birch (*Betula pendula* Roth), and rhododendron (*Rhododendron catawbiense* Michx.). MB had little effect on the percentage of explants forming shoots and the number of shoots formed per explant in birch and rhododendron, but dramatically reduced both responses in chrysanthemum. Therefore, the effects of MB must be evaluated for each species of interest prior to use [12].

This study aims to develop a protocol for sterilization techniques with MB compound in tissue culture propagation of *Casuarina inophyllum*, using stem cutting of nodal segments obtained from field grown plants.

## 2. Experimental methods

### 2.1 Location

The study was located at the tissue culture laboratory and nursery in Centre for Forest Biotechnology and Tree Improvement, Yogyakarta, Indonesia.

### 2.2 Plant Material and Culture Establishment

The sources for stemcutting of nodal segments were taken from naturalized mature male trees of *C. equisetifolia* that were estimated to be more than 20 years of age located in Goa Cemara beach, Bantul, Yogyakarta (latitude 07° 44' 04" – 08° 00' 27" and longitude. 110° 12' 34" – 110° 31' 08"). The stem cuttings preparations for explant source were done in nursery. Stem cuttings of *C. equisetifolia* were planted in soil and compost medium with 1:2 compositions. Two axillary buds per explant were excised from apical nodal segments.

### 2.3 Sterilization of explants

The explants without leaves were washed in soapy water for 5 min, rinsed in distilled water and gently shake with propamocarb hydrochloride 722g/l for 10 min, and then rinsed in distilled water again. All subsequent operations were carried out in a lamina air flow chamber and sterilized in 70% ethanol for 1 min, followed by sodium hypochlorite (NaOCl) 5% (v/v) plus 0.01% Tween-20 for 10 min under constant agitation. The explants were rinsed three times in autoclaved distilled water. We observed the explants sterilization effects of MB in liquid and solid medium in WPM basal medium [15] without hormone added. In liquid medium, explants were shaken with 8mg/l MB for 1 day, 2 days and 3 days shaking time. After being sterilized in liquid media, the explants were placed individually in a vertical position in flasks (10 cm high × 5 cm diameter), containing 10 ml of solid media supplemented with 0mg/l, 2mg/l, 4mg/l and 6 mg/l of MB, 30g/l sucrose and solidified with 8g/l agar. The pH of the media was adjusted to 5.8 prior to autoclaving at 121°C for 20 min. Explant contamination, microbial contaminant, mortality of explant and contaminant abundance were evaluated every day for 30 days in each treatment.

### 2.4 Regenerating shoots

In regenerating axenic culture, shoots were sub-cultured in the same basal medium without MB for 3 months (1 month per sub-cultured). At all *in vitro* stages, the explants were kept in a growth room at 20 ± 2°C by day under a 16h-photoperiod and a white fluorescent light. The percentages of swelling internode and sprouted axillary bud in axenic culture for each treatment were observed.

### 2.5 Statistical analysis

The experimental design for axenic culture in sterilization technique was completely randomized design (CRD) with 20 replications of each in 12 combination treatments (3 days shake × 4 MB concentrations), totalizing 240 explants per treatment. The data were statistically analyzed by using Microsoft Excel (Office 2007, Data Analysis – Descriptive statistics).

## 3. Results and discussion

### 3.1 Sterilization of explants

Sterilization was carried out since the preparation of *C. equisetifolia* stump cutting in nursery by spraying fungicides and bactericides. This is a zero stage, before explants processed from *ex vitro* were freed from microbial contamination and transferred to an *in vitro* environment in the first stage of plant tissue culture [16]. Young and soft nodal segments from axillary shoots (Fig. 1a) are part of plants with high cell division activity and have not been contaminated yet by many microbes. It will increase the explant regeneration and reduce sterilization efforts. To date there is no report on successful *in vitro* propagation of *C. equisetifolia* shoot tip cuttings from mature male trees (Fig. 1b). Shen et al. (2010) have established micro propagation of *C. cunninghamiana* using epicotyl

explants excised from seeds germinated in vitro, however the protocol had high contamination rates [17].

Liquid MB sterilization effects in three incubation times (Fig. 1c and Fig 1d) were determined empirically for each type of *C. equisetifolia* explants. A balance between concentration of disinfectant and time of treatment must be determined empirically for each type of explant because of phytotoxic effect [18]. Erwin & Wetzel (2002) had also noticed that high concentration of sterilization causing plant tissue death. Sterilization should not kill or break off the biological activity of explants, but the contaminants. Explants must be surface sterilized only by treatment with disinfectant solution at suitable concentrations for a specified period [9].



**Figure 1.** *C. equisetifolia* axillary shoot (arrow) from stem cutting as explant source (a), outside LAF sterilization of shoot tip cuttings taken from mature male trees with propamocarb hydrochloride (b), inside LAF sterilization of explants with ethanol followed by sodium hypochlorite (c) and shaking MB liquid sterilization before transfer to solid media with MB added (d).

Effects of different MB concentrations in WPM culture medium and different shaking times on explant contamination, microbial contaminant and contaminant abundance after 30 days of culture are shown in Table 1

**Table 1.** Effect of MB in liquid and solid WPM medium on *C. equisetifolia* explant contamination, microbial, mortality of explant and contaminant abundance after 30 days of culture (N = 20).

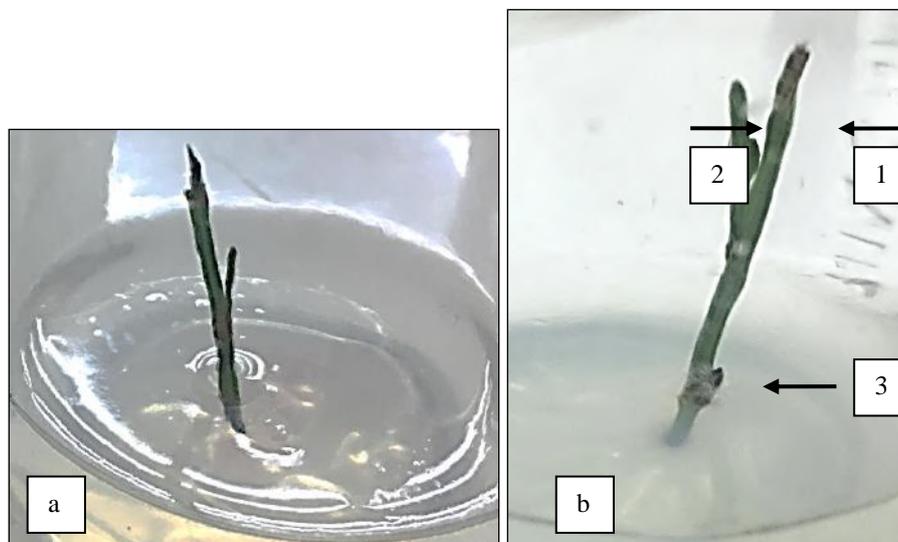
Time (day) in liquid medium	MB concentration (mg/l) in solid medium	Treatment	Explant Contamination (%)	Microbial	Mortality of Explant (%)	Contaminant abundance
1	0	P1	100 ± 0.1 <sup>a</sup>	Fungi and Bacteria	100 ± 0.1 <sup>a</sup>	++++
2	0	P2	100 ± 0.1 <sup>a</sup>	Fungi and Bacteria	100 ± 0.1 <sup>a</sup>	++++
3	0	P3	100 ± 0.1 <sup>a</sup>	Fungi and Bacteria	100 ± 0.1 <sup>a</sup>	++++
1	2	P4	74 ± 1.8 <sup>b</sup>	Fungi and Bacteria	28 ± 0.1 <sup>b</sup>	+++
2	2	P5	70 ± 1.9 <sup>b</sup>	Fungi and Bacteria	24 ± 0.1 <sup>b</sup>	+++
3	2	P6	69 ± 1.2 <sup>b</sup>	Fungi and Bacteria	20 ± 0.1 <sup>b</sup>	+++
1	4	P7	58 ± 1.1 <sup>c</sup>	Fungi	20 ± 0.1 <sup>b</sup>	++
2	4	P8	45 ± 1.0 <sup>d</sup>	Fungi	20 ± 0.1 <sup>b</sup>	++
3	4	P9	31 ± 1.1 <sup>e</sup>	Fungi	20 ± 0.1 <sup>b</sup>	++
1	6	P10	27 ± 1.4 <sup>f</sup>	Fungi	21 ± 0.1 <sup>b</sup>	++
2	6	P11	20 ± 1.0 <sup>g</sup>	Fungi	22 ± 0.1 <sup>b</sup>	++
3	6	P12	15 ± 1.5 <sup>h</sup>	Fungi	22 ± 0.1 <sup>b</sup>	+

Means followed by different letters are significantly different by Tukey’s test at p<0.05;++++ very high, +++ high, ++ low and + very low; MB:methylisothiazolinone biocide

Fungi and bacteria contaminated the explants in first day observation on medium without MB added at all shaking time treatments, while the contaminant began to appear after 5 days culture with 2 mg/l MB in all shaking time treatments, and only fungi contaminated for all higher concentration in all shaking times after 7 days culture. According to the Tukey’s test, 2 mg/l of MB resulted in no significant difference between the means. When considering the shaking time of exposure to the MB, the differences between the results of explants contamination were not significant between all shaking time in 2mg/l of MB. After 30 days of culture, concentration was highly effective in combating bacteria, where the concentrations of 4 mg/l and 6 mg/l could eliminate them. The highest concentration (6 mg/l) tested in longest shaking time (3 days) was more effective in reducing microbial contamination (P12), very low fungi contaminant. Another interesting result in this experiment was that all of MB concentrations and all shaking time treatments gave low percentage of explant mortality (below 30 %). It is possibly caused by different things due to the effective combination of MB in WPM. Thus it is still possible to increase the concentration of MB and the shaking time treatment to eliminate contamination and mortality of *C. equisetifolia* explant.

The best axenic culture of *C. equisetifolia* shoot tip cuttings after 3 days shaking sterilization in liquid WPM with 8 mg/l MB and being transferred to solid WPM with 6 mg/l MB are shown in Fig.2a. This modified technique showed the lowest contamination and mortality of explants. Microbial identification was not the focus of this research, general morphology of bacteria and fungi were observed for non-microscopic observation. Sterilization of explants is a key step in any plant tissue culture work, as the removal of all microorganisms, including bacteria and fungi. It is essential to attain successful initiation, growth and development of the cultured tissues in vitro, which otherwise would

be overwhelmed by the contaminants. The use of stem cuttings from field grown trees as a direct source of explants for the establishment of aseptic in vitro cultures is generally considered a major setback, especially with leaf canopy close to the ground [8]. The higher percentage of contamination in *C. equisetifolia* shoot tip cultures was due to nearer position of the branch as stem cutting material to the ground as source of contamination. In this study, contamination was successfully reduced in shoot tip explants using the modified sterilization protocol. However, easily necrotic and longtime regeneration of *C. equisetifolia* in vitro has caused a high contamination in each subculture of explant. It appears therefore for further research related to the problem.



**Figure 2.** Axenic culture of *C. equisetifolia* shoot tip cuttings sterilization in liquid WPM with 8 mg/l MB and after 14 transferred to solid WPM with 6 mg/l MB (a); axenic shoot proliferation after 30 days incubation on WPM (b): swelling internodes (1), single shoot formation from axillary buds at basal nodes (2) and sprouted lateral buds (3).

The explants should not lose their biological activity and only contaminants should be removed during sterilization. In the establishment of axenic cultures, primary consideration is given to the selection of suitable sterilizing agents and determination of the appropriate duration of explant exposure to the chemicals. As a result, the selected chemicals should be of a type that could easily be removed from the explant surface through repeated rinsing [20], while the duration of exposure is sufficient enough to ensure higher rates of explant survival after treatment. However, the uses 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 explants growth, as well as encouraging resistance buildup for the chemicals. It is essential to take the utmost care while selecting the chemical for use.

In our study, *C. equisetifolia* shows a good response in WPM medium without the addition of exogenous growth hormone. It showed that *C. equisetifolia* has high content of endogenous hormone (Fig. 2). The first indication of shoot tip explant response generally occurred within 15 days of culture initiation. Shoot tips changed from dark green color to a more obvious transparent green and the internode sections became swollen and barrel-shaped (Fig. 2b-1). Single shoot formation from axillary buds at basal nodes and sprouted lateral buds were visible within 30 days (Fig. 2b-2). Lateral bud of the more basal nodes was the first sprout in most cultures (Fig. 2b3). Nonresponsive explants turned into dark brown color and eventually died (shoot tip necrosis). Pahnwar (2005) reported that 6-benzyl

amino (BA) from 0 to 11.11 mM induced 38.5% to 73.86% of axillary bud sprouting from mature tree shoot tip explants of *C. equisetifolia*. However, BA at concentrations ranging from 0 to 16 mM failed to result in any shoot production from *C. cunninghamiana*[17]. The endogenous hormone levels have been regarded as critical for bud formation and even for plant regeneration in *in vitro* culture for many plant species [22]. There has been no report on endogenous hormones of axillary shoot explant from stem cutting during *in vitro* culture in *C. equisetifolia*. A better understanding of the relationship between endogenous hormone concentrations in the calluses and the bud formation competence will be helpful for *in vitro* propagation of *C. equisetifolia*. This study measured and analyzed the correlation between the endogenous hormone status of stem calluses and their ability to form buds.

#### 4. Conclusions

The results obtained in the present study provided a promising of MB for tissue culture sterilization of *Casuarina equisetifolia*. The best axenic culture of *C. equisetifolia* shoot tip cuttings was after 3 days shaking sterilization in liquid WPM added with 8 mg/l MB, and transferred to solid WPM with 6 mg/l MB. The modified technique for tissue culture sterilization of *C. equisetifolia* has lowest contamination and lowest mortality of explants. *C. equisetifolia* has a good response in WPM medium without the addition of exogenous growth hormone. It indicated that *C. equisetifolia* has high content of the endogenous hormone.

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#### 6. References

- [1] Mousavi SMM, Hosseini SZ, Resalati H, Mahdavi S, Garmaroody ER. 2013. Papermaking Potential of Rapeseed Straw, A New Agricultural-Based Fiber Source. *Journal of Cleaner Production*. 52: 420–424.
- [2] Lal PS, Sharma A, Bist V. 2013. Pine Needle - An Evaluation of Pulp and Paper Making Potential. *Journal of Forest Products & Industries*. 2(3): 42-47.
- [3] Samariha A, Kiaei M, Talaeipour M, Nemati M. 2011. Anatomical Structural Differences Between Branch and Trunk In *Ailanthus Altissima* Wood. *Indian Journal of Science and Technology*. 4(12): 1676–1678.
- [4] Turnbull JW. 1990. *Advances In Casuarina Research and Utilization*. In J. L. El-Lakany, M.H.; Turnbull, J.W.; Brewbaker (Eds.), *Taxonomy and genetic variation in casuarinas*. Cairo: Desert Development Center, American University in Cairo. pp.1-11 ref.35.
- [5] Geary TF. 1984. *Casuarina equisetifolia* L., *Part II-Species Description*. In *Tropical Tree Seed Manual*. U.S Department of Agriculture, Forest Service, University of Virginia, JA Vozzo (Ed.). pp. 378-381
- [6] Kannan CS, Warriar EV, Singh BG. 2015. Screening of Clones of *Casuarina Equisetifolia* for Pulping Traits Using Wood Fibre Characteristics. *International Journal of Current Research and Review (JCRR)*. 7(12): 64–71.

- [7] Shukry N, EI-Kalyoubi SF, Hassan ME. 1999. Pulping of *Casuarina glauca* with ASAM - An Environmental Friendly Process. *Journal of Scientific and Industrial Research*.58:799–806.
- [8] Webster SA, Mitchell SA, Achmad MH. 2003. A Novel Surface Sterilization Method for Reducing Fungal and Bacterial Contamination of Field Grown Medicinal Explants Intended For In Vitro Culture. Biotechnology Centre, UWI, Mona, Kingston.
- [9] Odutayo OI, Annusa NA, Okutade OO, Ogunsanwo YR. 2007. Determination of the Sources of Microbial Contaminants of Cultured Plant Tissues. *Plant Pathology Journal*. 6(1): 77–81.
- [10] Qin F, Shinozaki K, Yamaguchi-Shinozaki K. 2011. Achievements and Challenges in Understanding Plant Abiotic Stress Responses and Tolerance. *Plant & Cell Physiology*.9(9):1569–1582.
- [11] Felek W, Mekibib F, Admassu B. 2015. Optimization of Explants Surface Sterilization Condition for Field Grown Peach (*Prunus persica* L. Batsch. Cv. Garnem) Intended for In Vitro Culture. *Academic Journal*. 14(8): 657-660.
- [12] George MW, Tripepi RR. 2001. Plant Preservative Mixture™ Can Affect Shoot Regeneration from Leaf Explants of Chrysanthemum, European Birch, and Rhododendron. *HortiScience*.36(4): 768–769.
- [13] Daud NH, Jayaraman S, Mohamed R. 2012. An Improved Surface Sterilization Technique for Introducing Leaf, Nodal and Seed Explants of *Aquilaria malaccensis* from Field Sources Into Tissue Culture. *Asia-Pacific Journal of Molecular Biology and Biotechnology*. 20(2): 55–58.
- [14] Silveiraa SS, Cordeiro-Silvab R, Degenhardt-Goldbachc J, Quoirinb M. 2015. Micropropagation of *Calophyllum brasiliense* (Cambess.) from nodal segments. *Brazilian Journal Biology*.
- [15] McCown BH, Lloyd G. 1980. Commercially-Feasible Micropropagation of Mountain Laurel, *Kalmia latifolia*, by use of shoot-tip culture. *Combined Proceedings, International Plant Propagators' Society*.30: 421–427.
- [16] George EF, Debergh PC. 2008. Micropropagation: Uses and Methods. In George, EF, Hall MA (Eds.), *Plant Propagation by Tissue Culture Volume 1. The Background* (3rd ed., pp. 2–28). Springer Netherlands.
- [17] Shen X, Castle WS, Gmitter FG. 2010. In Vitro Shoot Proliferation and Root Induction of Shoot Tip Explants from Mature Male Plants of *Casuarina cunninghamiana* Miq. *Horticultura Science: A Publication of the American Society for Horticultural Science*.45(5): 797–800.
- [18] Wegayehu F, Mekbib F, Admassu B. 2015. Optimization of Explants Surface Sterilization Condition for Field Grown Peach (*Prunus persica* L. Batsch. Cv. Garnem) Intended for In Vitro Culture. *African Journal of Biotechnology*.14(8): 657–660.
- [19] Ervin GN, Wetzel RG. 2002. Effects of Sodium Hypochlorite Sterilization and Dry Cold Storage on Germination of *Juncus effusus* L. *Wetlands*. 22(1): 191–195.
- [20] Pahnwar F. 2005. Acclimatization and Establishment of Micropropagation Plant.
- [21] Seth R, Kendurkar S., Nadgauda R. 2007. In vitro Clonal Propagation of *Casuarina equisetifolia* Forst. from Mature Tree-Derived Explants. *Current Science*.92(3): 287–290
- [22] Ali A, Ahmad T, Abbasi NA, Hafiz IA. 2009. Effect of Different Concentrations of Auxins on In Vitro Rooting of Olive Cultivar Moraiolo. *Journal Botany*.41(3):1223–1231.