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A STUDY ON IMPACT OF AUXIN AND ELICITORS ON TISSUE CULTURE AND PROLIFERATION OF *Nigella sativa* L.

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ABSTRACT

Nigella Sativa is an annual garden flowering plant, belonging to the buttercup family Ranunculaceae. It is native to southern Europe, North Africa and Southwest Asia, where it is found on neglected, damp patches of land. Black cumin seed contains oil; protein; alkaloids such as nigellicimine and nigellidine; kinons such as timokinon and saponin; and Volatile essence. It is used as a homeopathy cure for respiratory conditions such as asthma, cough, bronchitis, etc. Tissue culture is one of the techniques which are used in biotechnology and plant breeding and it is among the most powerful materials for propagation and breeding. Accordingly, a study was conducted in the tissue culture laboratory of the Laboratory Complex, Islamic Azad University, Science and Research Branch, Tehran, to investigate the effect of different hormones densities on internode and hypocotyl regarding different characteristics like the number of shoots and roots, their lengths, their fresh and dry weights, the fresh and dry weights of callus and its volume in March 2015. Hormone treatments included BA with the densities of 0.5, 1, and 2 µM, NNA with the densities of 0, 0.5, and 1 µM, and SA with the densities of 0 and 1 µM. The results indicated that the maximum number of shoots and roots, length of roots, dry weight of roots, fresh weight of shoots, fresh weight of callus and its volume were observed in the hormone density of BA=2, NAA=1, and SA=1. In addition, the longest shoot was observed in the hormone density of BA=0.5, NAA=0, and SA=0. No statistically significant interaction was identified between the hormone treatment and the dry weight of callus, the fresh weight of root, and the dry weight of shoot. Also, there wasn't any statistically significant interaction between hormone treatment and the sampling places on the characteristics on the probability level of 5%.

Keywords: plant growth regulators, proliferation, callus, tissue culture, medicinal plants.

1. INTRODUCTION

Old knowledge of medicinal properties of plants is beyond historical memory. An important reason for dating is rooted beliefs of different peoples on the use of medicinal plants (Beigi, 2005).

Interest in plants, due to the effects of plant-derived drugs and interest in natural products, has expanded. Using natural products as an alternative to conventional treatment in the health and treatment of various diseases has increased in recent decades because of the side effects of conventional drugs (Dattner, 2003).

Medicinal plants are alternative therapies or are the sole effective treatments. Most medicinal plants and their constituents have various health effects including antioxidant, anti-inflammatory, anti-cancer, anti-microbial and safety regulators (Miller et al., 2004; Huffiman, 2003; Dattner, 2003). Medicinal plants are a major source of pharmaceuticals that are used to treat various diseases. In addition, the active substances of plants, especially perfumes and essential oils, have multiple applications in different industries, including cosmetics; household chemicals such as shampoo and soap; and perfume, so that without the presence of the active substances, manufacturing and supplying many of the products mentioned above is not possible (Beigi, 2005). In the past, the medical plants were primary source of healing and were widely used by the people. With the arrival of synthetic drugs, the use of natural materials reduced, but recently human stem familiarity with the properties and beneficial effects of natural medicines and harmful effects of chemical drugs has led to increased use of this material (Beigi, 2005).

Among the medicinal plants is *Nigella sativa* L. that is widely in different parts of the world and has been used as a natural medicine for many diseases. Seeds and oils in this herb have a long history of medicinal use in traditional medicine, as well as food industry. In Islam, the *Nigella sativa* L. is a great healer. Due to diverse use of *Nigella sativa* L., much focus has been made on this plant. For example Black Cumin seeds are a source of oil and protein, especially in industry and in the production of many oleo chemical compounds used (Sharma *et al.*, 2009).

Nigella sativa is a diploid plant with chromosome number of 2n = 12 (Iqbal *et al.*, 2010) and genus of Nigella includes 20 species covering a wide range of geographical locations from the Mediterranean region to West Asia (Riaz *et al*., 1996), also in Iran almost 8 species have been established (Mozaffarian, 2003). Some species of Nigella such as N. arvensis and N. damascene are subjects of more attention in terms of medicinal properties (Burits and Bucar, 2000). The only cultivated species by farmers is *Nigella sativa* L. or black culmin (D'antuono *et al*., 2002).

Nigella sativa is also known by other names in the culture of different countries. For example, in English: Caraway; in Sanskrit: Akhuji, Kalajyra; in Arabic: Habat Al-Sawda; in the United States: Cumin, in Urdu and Hindi: Kalonjy and also in Persian: Shoniz; Siah Daneh and Indian Kamoon. In some books on traditional medicine, *Nigella sativa* L. is called by other names such

as Sumer (Goreja, 2003; Samsam Shariat, 1995; Zargari, 1993).

2. LITERATURE

Duke (Duke, 1982) mentioned the following conditions for the growth of the *Nigella sativa* L.: a minimum temperature of 7.8 ° C, the optimum temperature of 13 ° C, maximum temperature 21°C, minimum required annual rain 430 mm, 790 mm optimum rainfall and maximum 1530 mm of annual rainfall, minimum acidity (pH) of the soil 6.9, and optimum pH of 8.2.

Hajzadeh (2006) studied the effects of alcoholic extract of Nigella sativa on kidney stones resulting from ethylene glycol in mice and concluded that alcoholic extract of Nigella sativa was effective in preventing the accumulation of crystals in calcium oxalate and in kidney stone crushing, and this is due to anti-inflammatory and anti-fat properties of *Nigella sativa* L. and its interference in the process of apoptosis.

Mandal AND Maity (Mandal and Maity, 1993) examined the effects of *Nigella sativa* L. immersion in gibberellic acid and observed that immersion in gibberellic acid at 80 ppm for 24 hours a day increased germination in the laboratory from 50 percent to 90 percent. It was also found that the highest percentage of germination occurred in complete darkness.

Salehi (2001) studied planting season of this plant in Northern Fars and observed that the autumn crop yield was far more satisfactory than spring planting. However, in the autumn cultivation, plant didn't appear on the soil surface in autumn and winter and spent these seasons as a seed.

Asgari *et al*. (2008) showed that *Nigella sativa* L. has no impact on white blood cells, erythrocytes, hemoglobin, hematocrit and fibrinogen, and only results in a statistically significant amount of platelets and may thus increase coagulation. Therefore, except for the increase in platelets, *Nigella sativa* L. had no impact on other hematologic indices.

In study of impact of different irrigation treatments on quality of *Nigella sativa* L. Bannayan (Bannayan *et al*., 2006) showed that Nigella Sativa can tolerate shortage of water at all times except at formation of seed. The lowest yield of seed occurred when irrigation was stopped at flowering and the number of seeds in each plant is the main factor that is affected at that time; however, oil concentration is not affected.

Shah (Shah, 2008) studied the effect of use of GA3 (0, 10^{-4}, 10^{-5}, 10^{-6} mole) on Nigella response to different levels of N (0, 176, 264, 325, 422 mg of nitrogen per pot) in India. N-fed plants showed a significant increase in the number of capsules and grain yield and nitrate reductase activity in addition to protein and oil yield, especially when taking 325 mg of nitrogen per pot. However, in comparison to all parameters, seed's oil content reduced at all treatments and use of a combination of 325 mg of nitrogen and 5-10 mole GA3 was introduced as the best treatment.

In an interesting experiment conducted by Paramnik (Paramnik *et al*., 2007), the effect of spraying solution of non-ionic water of mole gibberellic acid GA3, AB and Zea and/or kinetin (KIN) 40 days after cultivation (growth period) on the growth and yield of *Nigella sativa* L. was studied. Applications of gibberellic acid mole had the greatest effect among hormones on growth of root and stem and their length, stem weight, dry weight, number of leaves, leaf area and number of branches at 70 days after planting. Using mole gibberellic acid resulted in the highest number of capsules and grain yield.

Safarnejad (Safarnejad *et al*., 2007) in Mashhad conducted a study on the effect of salinity on *Nigella sativa* L.; this experiment was done in hydroponic conditions with four replications in the germination and seedling stages and with three replications at seedling stage. Salinity levels included 0, 50, 100, 150, 200 mol calcium chloride per cubic meter. The results showed significant differences for germination, seedling vigor, root length and biomass and increase in salinity. Nigella sativa showed high tolerance to increased salinity in seedling stage compared to germination stage.

Faravani *et al.* (2006) collected native Nigella from all parts of Iran and cultivated them in Khorasan. Significant differences was observed in terms of plant height, grain weight, grain yield and number of branches, vascular bundles, phloem and wood weight and diameter of the capsule. Only biological yield and harvest index and number of branches were correlated in accordance with changes in yield. Biological yield and HI and lateral branch had maximum heritability and genetic gain.

Shah and Samiullah (Shah AND Samiullah, 2006) conducted an experiment in which the impact of solution spraying of non-ionic water and 10^{-5} mole gibberellic acid or kinetin (KIN) 40 days after cultivation (growth stage) on the growth and yield of *Nigella sativa* L. was studied. Applying 5-10 mole gibberellic acid increased the number of capsules and grain yield. Applying GA3 in concentrations of 5-10 was more effective than kinetine in increasing the length of the branches, leaves of the plant, dry weight, total leaf area and the number of branches observed at 70 days after planting. Applying 10^{-5} mole gibberellic acid increased number of capsules and yield of seed.

Parvardeh (Parvardeh *et al*. 2002) reported that Nigella sativa may also be useful in epilepsy. They also reported the anticonvulsant effect of Nigella in 2013, which is mainly due to stimulation of opioid receptors in the central nervous system. Roghani (Roghani et al. 2006) investigated the analgesic effect of *Nigella sativa* L. and concluded a significant reduction in the amount of pain in diabetes mellitus.

3. METHODOLOGY

3.1 Time of experiment

This research was carried out in the laboratory in a factorial fashion and with a completely randomized design with 12 treatments and 4 replications on *Nigella sativa* L. in May 2015.

- The first factor: Auxin compound including three levels:
  a: BA=0.5µm ،NAA=0µm, b :BA=1µm ، NAA=0.5µm, c :BA=2µm ،NAA=1µm
- The second factor: the elicitor salicylic acid in two levels:
  a) SA=0µm b) SA=1µm
- The third factor: the type of explants at two levels:
  a) hypocotyle b) internode

3.2 Place of the test

The study was conducted in Laboratory (tissue culture and horticulture laboratory), Islamic Azad University, Science and Research Branch.

3.3 Preparation of plant materials

An important aspect in the success of culture of tissue culture is to have healthy explants. That is why we tried to use the best of Nigella sativa seeds as collected from Kashan.

3.4 Laboratory equipment and sterilization of laminar

We washed all devices and then rinsed with distilled water and drained and dried them before autoclave. Glass equipment such as Petri dishes, beaker, calibrated cylinder, test tube and other devices capable of autoclaving such as forceps, scalpel, knife, etc. were covered in foil and were autoclaved at 121°C and pressure of 2.1 Atm for 20 minutes. After autoclaving, equipments were transferred to the hood.

To start to work under laminar, laminar must be turn on 20 minutes before starting the work and walls of laminar were cleaned and disinfected with 70% ethanol. Instruments including forceps, scalpel, knife and sterilized paper, glass jam and tubes were sterilized under the hood. Hands were washed before working using soap and 70% alcohol and after every contact with samples, the forceps and scalpel were placed in 70% alcohol and then were sterilized using bonfires; disinfecting the work surface was conducted using 70% alcohol.

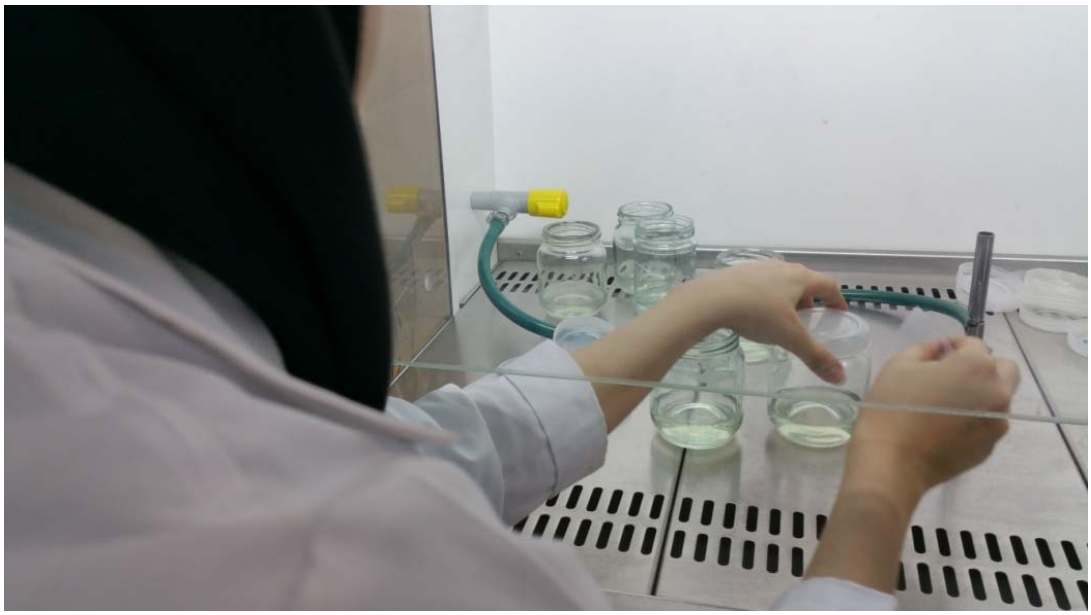

Figure-1. Glass containers and laboratory equipment sterilization and preparation of culture medium.

3.5 Medium

MS medium for the growth of seeds and MS medium containing different concentrations of hormones and elicitor for the growth of explants were considered.

3.6 Laboratory operations

3.6.1. Disinfecting samples

To disinfect explants, the seeds were poured into the glass plate, washed with dishwashing liquid, then rinsed with water and rinsed several times with distilled water. Then the seeds were placed in distilled water for 1 hour to be dehydrated. The seeds were transferred to laminar hood and were sterilized using 3% sodium hypochlorite for 15 minutes by shaking, and then were rinsed with distilled water, were cultured in MS medium.

Figure-2. Disinfection and planting seeds under laminar hood.

3.6.2.1. Final vitro

To produce 1 liter of culture medium, using calibrated pipettes, certain amount of salts, vitamins and iron were taken and placed in 1-liter calibrated cylinder, 30 g of sucrose was added to the solution. Then the volume of solution in calibrated cylinder was raised to 1000 ml using distilled water, and cylinder was placed on a magnetic stirrer. The pH solution was set between 5.6 and 5.8 using normal HCl 1 and normal NaOH 1. At the end, agar was added at 4 grams/liter, the mouth of the flask was sealed with cotton and aluminum foil, then the culture medium was autoclaved at a temperature of 121°C and pressure of 1.2 atmospheres for 90 minutes.

3.6.3. Preparation and cultivation of explants

After transferring the explants to the laboratory, healthy seeds got isolated and initially cleaned with dishwashing liquid and then rinsed with distilled water three times, and immersed in distilled water for 60 minutes in the beaker, and then for final disinfection transferred to the laminar hood (Figure 3-1).

Ready explants under laminar hood were disinfected initially with 3% sodium hypochlorite solution treatment (60 ml of sodium hypochlorite + 40 ml of distilled water) for 15 minutes by shaking, then rolled in sterile water for one minute. Seeds were dehydrated and then placed in culture. Petri dishes containing MS medium and Nigella sativa seeds were tightly sealed and placed in an incubator. Explants remained there for three weeks so contamination surfaces during this period and proper disinfection treatment was chosen for continuation of the experiment.

3.6.4. In vitro culture operations

After the formation of seeds and seedling growth, hypocotyl and internode explants were obtained and cultured in MS medium with hormones and elicitor. Four explants contained in each bottle of jam. Three weeks later, explants started rooting, developing callus and having longitudinal growth. After 28 days, subculture of explants was performed and the used iron was changed from EDTA to EDDHA to ensure better and greener growth of explants.

3.6.5. Incubator's conditions

6000 lux light intensity was used in the incubator, which was supplied by cool fluorescent lighting, average temperature of 25 ° C., relative humidity of 70% and photoperiod of 16 hours of light and 8 hours of darkness.

Figure-3. Plates containing seeds inside the incubator.

3.7 Traits tested

After the treatment period, quantitative traits such as number of shoots, shoot length, number of roots, root length, root fresh weight, root dry weight, shoot fresh weight, shoot dry weight, callus volume, callus fresh weight, callus dry weight were measured for all explants. Digital scale was used to measure the weight up to four decimals. The samples were then placed for 72 hours at 60°C oven. The dry weights were measured using a digital scale.

A ruler was used to measure the length of shoot and root length.

Callus volume was measured using destructor method. For this purpose, the 100 ml cylinder was filled with distilled water to 50 cc and samples dived into it using a small forceps tip so that the water covered the entire surface of the sample and the observed increase in volumes measured.

3.8 The methods and data analysis tools

A) Descriptive statistics

In this section, phenotypic variation coefficient, mean, variance, standard deviation, standard error,

minimum, maximum and range of recorded data obtained from the evaluation of explants were analyzed.

B) Analysis of correlation coefficient

Using correlation coefficient, bilateral relationship between different traits was measured and traits the choice of which could increase the efficiency were identified. For this purpose, and for estimating simple phenotypic correlation coefficients between traits, analysis of correlation coefficients between evaluated traits was conducted by selection of Pearson r and parametric correlation analysis on two levels of 0.01 and 0.05.

During the study, prior to analysis, the normality test was first done on the raw data obtained and the required conversion was done for data normally distributed. It should be noted that the tables of variance analysis are presented based on the converted data. However, the mean difference and the mean squared are expressed in terms of converted data. ANOVA analysis methods were used for data analysis and to compare the mean, also Duncan test was used at 95% level of significance. In addition, for drawing graphs and charts and data organization, MICROSOFT EXCEL 2013 software was used.

4 RESULTS

4.1 Effect of different hormonal treatments on different traits of *Nigella sativa* L. in the sampling sites

4.1.1. Effect of different hormonal treatments on root length in internode

There was no statistically significant difference between different hormonal treatments in terms of root growth part. As seen, the largest root length is related to treatment 6 and the shortest is in treatments 2, 3 and 5.

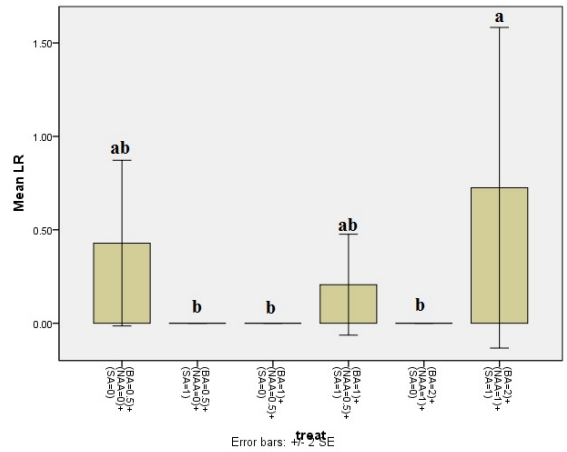

Figure-4. Mean of hormone treatments on length of root.

4.1.2. Effect of different hormonal treatments on root fresh weight in internode

No statistically significant difference was observed between different hormonal treatments on root length.

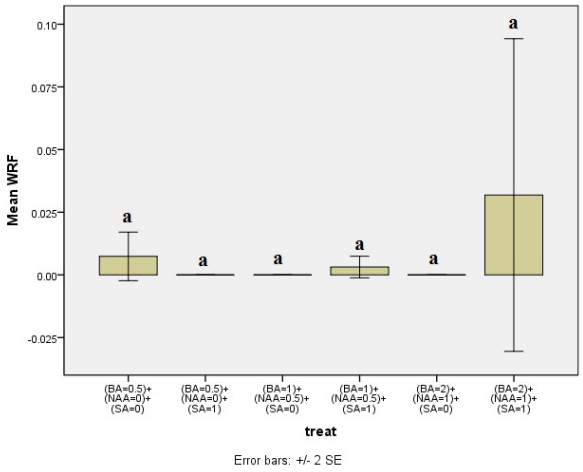

Figure-5. Mean of hormone treatments on fresh weight of root.

4.1.3. Effect of different hormonal treatments on root dry weight in internode

No statistically significant difference was observed between different hormonal treatments on root length.

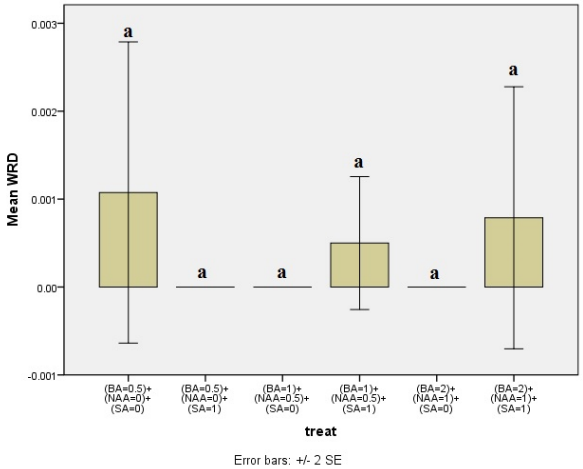

Figure-6. Mean of hormonal treatments on dry weight of root.

4.1.4. Effect of different hormonal treatments on root fresh weight in internode

No statistically significant difference was observed between different hormonal treatments on root length.

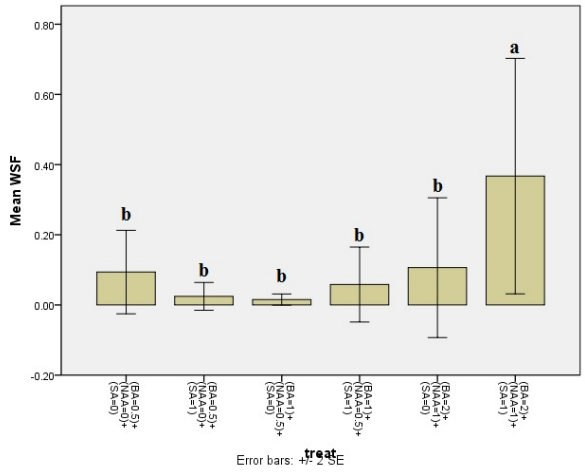

Figure-7. Mean of hormonal treatments on fresh weight of shoot.

4.1.5. Effect of different hormonal treatments on shoot dry weight in internode

No statistically significant difference was observed between different hormonal treatments on root length.

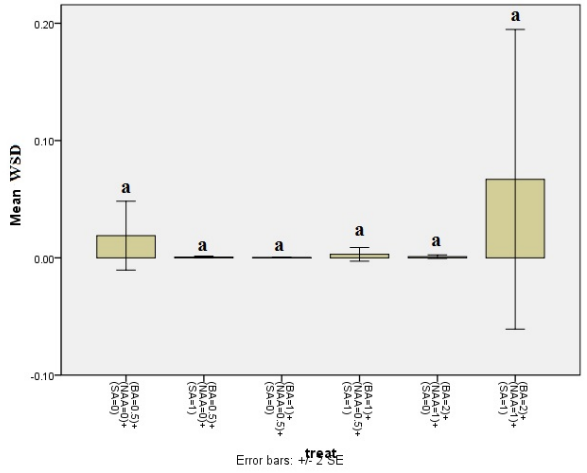

Figure-8. Mean of hormonal treatments on shoot dry weight.

4.2 The overall effect of the explants types on the characteristics

4.2.1. Effect on the root number of explants treated with BA=1+NAA=0.5+SA=0

Study of effect of sampling site on root number or regeneration in specific hormonal treatment showed that there was no statistically significant difference between the sampling site of internode and hypocotyl on the root number.

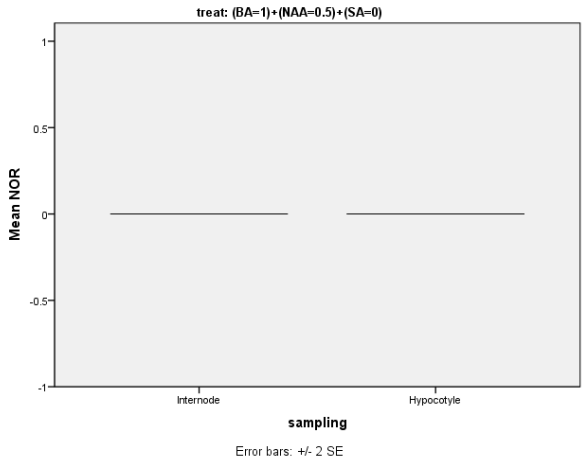

Figure-9. Mean of root number on type of explants treated with BA=1+NAA=0.5+SA=0.

4.2.2 Effect on root number of explants treated with BA=1+NAA=0.5+SA=0

Study of effect of sampling site on root number or regeneration in specific hormonal treatment showed that the explants prepared from hypocotyl were more efficient in generation of root compared to those prepared from internode.

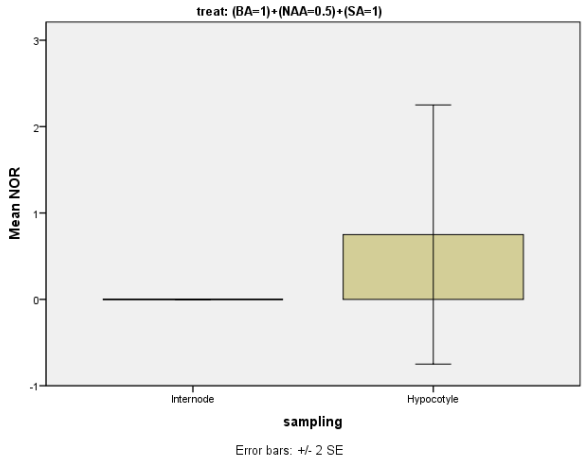

Figure-10. Mean of number of roots on type of explants treated with BA=1+NAA=0.5+SA=1.

4.2.3. Effect on the root number of explants treated with BA=2+NAA=1+SA=0

Study of effect of sampling site on the root number or regeneration in specific hormonal treatment showed that there was no statistically significant difference between the sampling site of internode and hypocotyl on the root number.

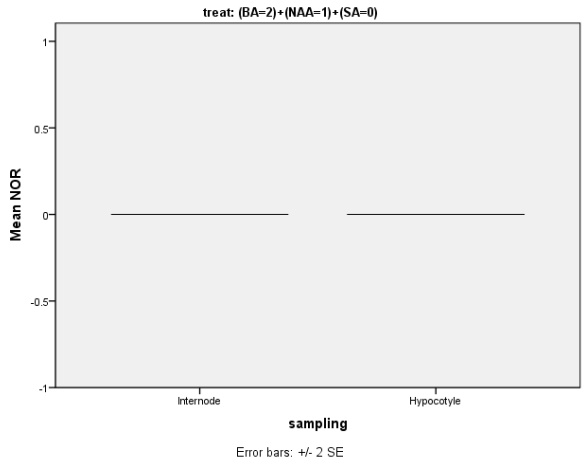

Figure-11. Mean of root number on type of explants treated with BA=2+NAA=1+SA=0.

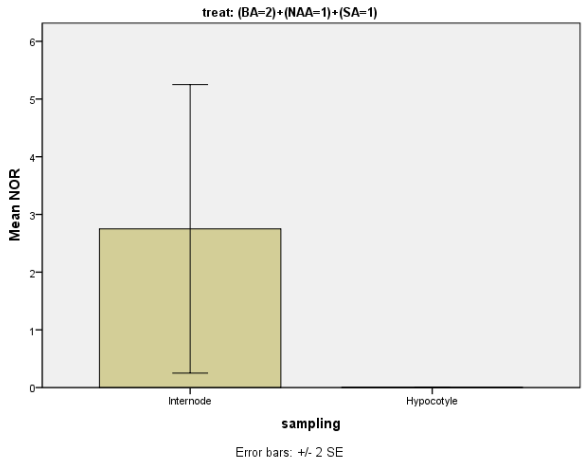

Figure-12. Mean of root number on type of explants treated with BA=2+NAA=1+SA=0.

4.2.4. Effect on the root number of explants treated with BA=2+NAA=1+SA=1

Study of effect of sampling site on the number of roots or regeneration in specific hormonal treatment showed that the explants prepared from internode were more efficient in generation of root compared to those prepared from hypocotyl.

4.3 Effect of type of explants on different traits of Nigella Sativa

4.3.1. Effect of type of explants on shoot number

At different concentrations, there was statistically significant difference in type of internode explant on the shoot number. The best hormonal treatments related to the internode was observed in BA=2, NAA=1, SA=0. At different concentrations of treatments, there was statistically significant difference in type of hypocotyl explants on the shoot number. The best treatments in hypocotyl were observed in BA=1+NAA=0.5+SA=0 and BA=0.5+NAA=1+SA=1.

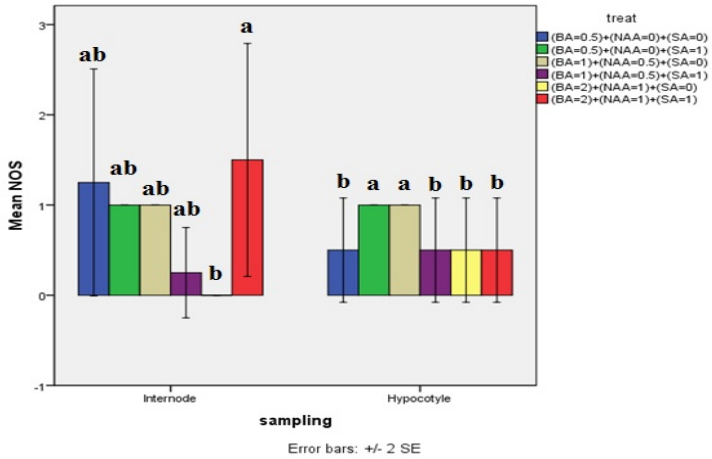

Figure-13. Different concentrations of treatments on mean number of shoots on the type of explant.

4.3.2. Effect of type of explants on shoot length

Different concentrations of treatments on type of internode explant on shoot length were statistically significantly different. The best treatment was observed in BA=2, NAA=1, SA=1. Different concentrations treatments in type of hypocotyl explants on shoot length were not statistically significantly different. The best hormonal composition was observed in BA=0.5+NAA=0+SA=0.

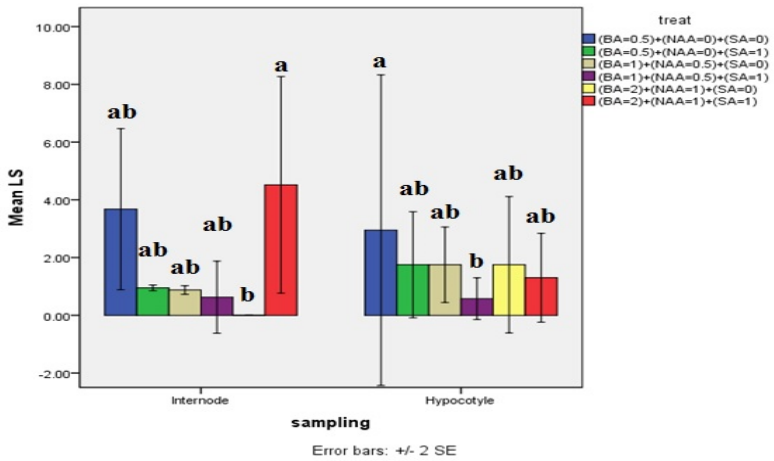

Figure-14. The different concentrations of treatments on mean of length of the shoot on the type of explant.

5. DISCUSSIONS

Tendency to produce medicinal and aromatic plants is increasing due to great demand for natural products in the world (Carruba *et al*., 2002), so that the twentieth century is called the century of return to nature and use of herbal medicines (Golshadi *et al.*, 2002). Yield increase may also be obtained through genetic manipulation. It's been predicted that the world's population in 2050 will amount to more than 10 billion, so the need to improve the performance of cultivars and local varieties will be in place. One of the possible ways to achieve this goal is to increase yield through tissue culture (soma clonal variation) that occurs without too much involvement of genetic manipulation. Due to the intervention of biotic and abiotic factors in the process of tissue culture, irregular variation in plants regenerated from this process is considerable (Bano *et al*., 2005).

Plant tissue culture is an important tool in basic and applied research and the production of seedlings and plants and it is very important on a commercial scale (Bagheri and Azadi, 2002). To succeed in tissue culture, medium composition is very important. Hormones such as Auxins, Cytokinins and Gibberellic that are used to control cell growth and division can be added to the culture medium at the right time, which have an important role in the formation of callus. Some parts of cultivated plants need Auxin to produce callus and some others only need Cytokinin while most cultures need both. Optimal Formula of medium varies depending on the species, type of genotype within the species and origin and the age of tissue culture (Ghasemi Bezdi and Ahmadi, 2010).

In most experiments in this field, culture has been conducted in base medium of Morashige and Skoog (Morashige and Skoog, 1962) at different ratios of plant hormones. In most researches, when cutting explants, due to the high amount of plant phenols and browning reaction of callus, parenchymal cell mass is formless and undifferentiated, with a thin cell wall whose origin is dividing cells of the mother plant tissue. The most important feature of callus is that, this cellular mass has the necessary potential for organogenesis, embryogenesis and complete plant production. Callus growth of woody perennials is usually slower than herbaceous ones in micro propagation. The level of plant growth regulators, such as Auxin, Gibberellin and Cytokinin is an important factor that affects the formation of callus in medium (Bagheri & Azadi, 2002).

Sunita *et al*. (2005) studied the length of shoots in Podophyllum Hexandrum in the presence of hormone BA at a concentration of 0.5 to 2 mg. They found that increasing the concentration of BA reduced the length of the shoot, which the results were consistent with our results. Negative effects of high concentrations of BA on length of shoot is probably due to the fact that increasing external Cytokinin concentration inhibits the synthesis of RNA and proteins, due to its antagonistic impact, so the synthesis of indole acetic isoenzymes in the cell are prevented, which ultimately reduces Auxin levels in cell, followed by reduced growth and cell division. Lim et al. (2009) have emphasized that the hormones added to the culture medium can have antagonistic and synergistic impact on endogenous hormone and thus their performance.

The results of the effect of hormones on shooting in the Hypericum showed that the presence of Cytokinin BA increased shooting in lateral buds and dominance over apical dominance of plant, which were consistent with our results. Further, BA combined with NAA showed a significant increasing impact on shooting. BA hormone concentration increase from 0.3 to 0.6 in combination with NAA increased the number of shoots; however, increasing concentrations of BA without the presence of NAA reduced the number of shoots per sample.

According to the results, it seems that NAA has an effective role in helping BA in the mechanism of apical dominance. Ghanti et al. (Ghanti *et al*., 2004) reported that Cytokinin, especially BA, resulted in awakening of lateral buds by overcoming the apical dominance. They found that compared to the effects of BA alone and in

combination with NAA, a combination of two hormones significantly increased the number of shoots per sample.

6. CONCLUSIONS

The highest number of shoots, number of roots, root length, root dry weight, shoot fresh weight, callus fresh weight and the volume of Callus were observed in treatment BA=2+NAA=1+SA=1.

The longest shoot was observed in hormonal level BA=0.5+NAA=0+SA=0.

Statistically significant interaction between various hormonal treatments and the callus dry weight, shoot dry weight and root fresh weight were not observed.

Statistically significant interaction between treatment and sampling site was not observed at 5% probability level.

7. SUGGESTIONS

In line this study, the followings are recommended:

The effect of other types of Auxin, such as NAA and D 2, 4 can be used for callus induction of various explants.

Given the different effects of Cytokinin, use of other types of them such as kinetin, TDZ and 2-ip is recommended.

Given type of explant has an important role in callus induction and regeneration, use of leaf explants is recommended.

Since different elicitors have different effects in different plants, so other use of other compounds such as Jasmonate and polyamines is recommended.

Constituent compounds of medium function as factors affecting callus induction explant regeneration and growth; thus it is recommended that different cultures be used.

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