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Water Stress Hinders *In Vitro* Regeneration of Plants

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Abstract

Plants could be propagated vegetatively via small parts of living tissue called as 'explant' on growth mediums under sterile conditions. Plant cell has the ability of forming whole fertile plant which is called 'totipotency', under *in vitro* culture conditions. High-frequency shoot regeneration is one of the main aims of *in vitro* culture and it is a prerequisite to guarantee the success in transformation studies and in clonal propagation of plants. It is well known that growth regulators in culture medium and the type of explant affect *in vitro* regeneration frequency significantly. In this chapter, the importance of tissue water content on *in vitro* culture response is discussed. Increasing water content of the explant before culture initiation gives rise to increased regeneration capacity. On the other hand, increasing the tissue's osmotic pressure enables the explant to intake water, all solutes and growth regulators from the growth medium which results in high-frequency shoot regeneration. However, tissues with lack of water are usually not successful in regenerating a satisfactory amount of shoots. The effect of water deficiency on explant's regeneration capacity and the methods to overcome this problem are discussed in this chapter.

Keywords: Plant *in vitro* culture, regeneration capacity, water, stress, growth

1. Introduction

Plant tissue culture includes techniques to propagate plants via somatic cells by using small parts called as explant on artificial growth mediums under sterile conditions. Shoots and roots are regenerated from explants, and consequently, the whole fertile plants are reconstituted under certain cultural conditions. Plant tissue culture belongs to totipotency meaning that a whole plant can be reproduced from a single cell in growth medium. Obtaining high-fre-

cy shoot regeneration is one of the major objectives for tissue culture studies that is also a prerequisite for an efficient transformation system and a clonal production of plants with interesting flowers and fruits massively for ornamental aims.

Plant tissue culture techniques have certain advantages over traditional propagation methods. Via tissue culture methods, thousands of mature plants having desirable traits such as good flowers, fruits and odor can be produced in a short time; endangered species which cannot propagate in native environment can be cloned easily by vegetative parts; genetically identical plants can be produced with large quantities; genetically modified plants can be regenerated from cultured cells; production of disease-, pest- and pathogen-free plants increase the plant production; and plants having seed germination and growing problems can be easily produced.

Plant growth regulators as media components affect the shoot regeneration capacity of explants. Tissue culture studies have tried to determine correct combinations of auxins and cytokinins for high-frequency adventitious shoot regeneration for related genotype. However, determination of optimum levels of auxins and cytokinins in growth medium is not the only way of increasing shoot regeneration capacity. It is reported that regeneration capacity of explant could be increased by adjusting the concentration, temperature and application period of NaOCl solutions used for surface sterilization [1] and manipulating physical microenvironment by altering distances among explants cultured resulted in increased shoot regeneration capacity [2]. Recently, it is noted that water capacity of the tissue affects explant's regeneration capacity significantly [3–5].

Source of life is based on water on the earth. Living is limited in a large proportion of terrestrial ecosystems according to water availability. The water content in an actively growing plant can be as much as 95% of its live weight. Water is needed in a plant for photosynthesis. Carbon dioxide and oxygen which is required for photosynthesis cannot be used by plant if they are not soluble in water. For this reason, water is the main factor for plant's existence and growth. Mineral ions such as potassium (K^+), sugars (glucose and sucrose) and amino acids are dissolved in water.

The decrease in growth, yield and quality by water stress has been reported in field conditions [6,7]. Plant survival is guaranteed by germination and seedling establishment and they are very important phases of plant life. Germination ratio diminishes with decreasing external water potential and there is a critical value of water potential for each species below which germination will not occur [8].

This chapter is aimed to show the effects of water deficiency in tissue on shoot regeneration capacity of the explants cultured under *in vitro* conditions. Moreover, increasing shoot regeneration frequency of explant by enhancing water content of the tissue is another issue this chapter focused on. All the results given here were based on three research studies.

2. The effect of increasing tissue water content on *in vitro* regeneration

It was reported that tissue water content affected explant's shoot regeneration capacity significantly [3]. Yildiz and Ozgen [3] have conducted a study to evaluate the effect of tissue

water content on regeneration capacity of hypocotyl explants of flax (*Linum usitatissimum* L.). In the study, water-treated and non-water-treated hypocotyl explants of three flax cultivars ('Madaras', '1186 Sel.' and 'Clarck') obtained from Northern Crop Science Laboratories, North Dakota, USA, were compared with regards to fresh and dry weights, shoot regeneration percentage, shoot number per explant, shoot length and total shoot number per Petri dish. Sterilized seeds were germinated on a basal medium containing the mineral salts and vitamins of Murashige and Skoog (MS) [9], 3% (w/v) sucrose and 0.7% (w/v) agar. Hypocotyl segments of 5 mm length were excised from 7-day-old seedlings. Some hypocotyls were submerged in sterile distilled water and shook gently for 20 min before they were placed on growth medium for regeneration, while the others were directly cultured on MS medium containing 1 mg l⁻¹ 6-benzylaminopurine (BAP) and 0.02 mg l⁻¹ naphthaleneacetic acid (NAA) to regenerate. It is clear according to the results that there were sharp and statistically significant differences in all cultivars between water-treated and non-water-treated tissues related with all the characters examined (**Figure 1**).

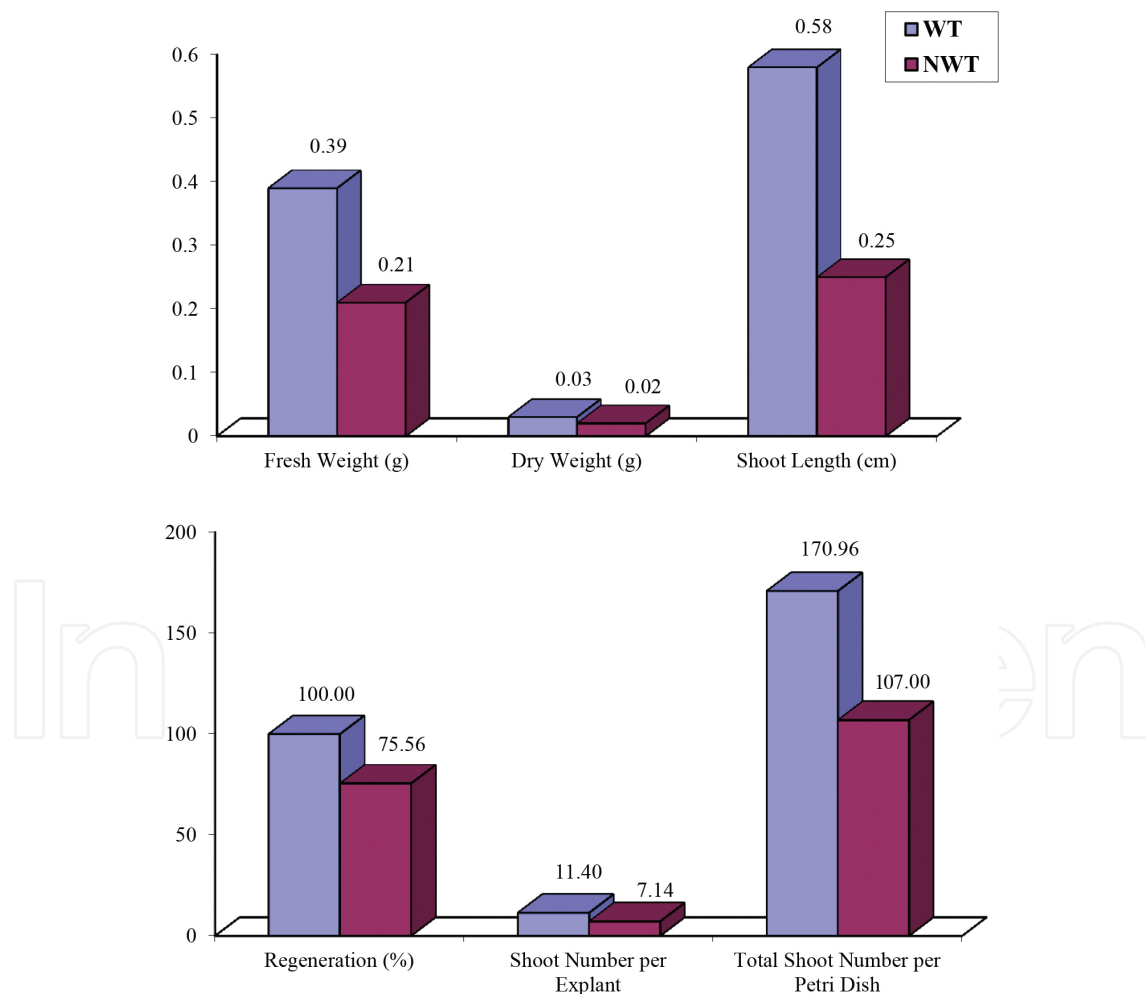


Figure 1. Tissue culture response of water-treated (WT) and non-water-treated (NWT) hypocotyl explants of three flax cultivars ('Madaras', '1186 Sel.' and 'Clarck') 6 weeks after culture initiation on MS medium containing 1 mg l⁻¹ BAP and 0.02 mg l⁻¹ NAA. Value on each the bar is the mean of three cultivars [3].

In the study, all explants were regenerated in water treatment application while only 75.56% of explants formed shoots in non-water treatment application. Water-treated explants had the highest fresh and dry weights compared to non-water-treated ones at the end of the culture (Figure 2(a) and (b)). Shoots grown from water-treated explants were more vital and well grown (Figure 2(c)) than the ones recovered from non-water-treated explants (Figure 2(d)). The highest shoot number per explant and total shoot number per Petri dish were obtained from the water-treated hypocotyl explants as 11.4 and 170.96, respectively. On the other hand, non-water-treated explants gave rise to only 7.14 shoots per explant and 107 shoots totally per Petri dish (Figure 1).

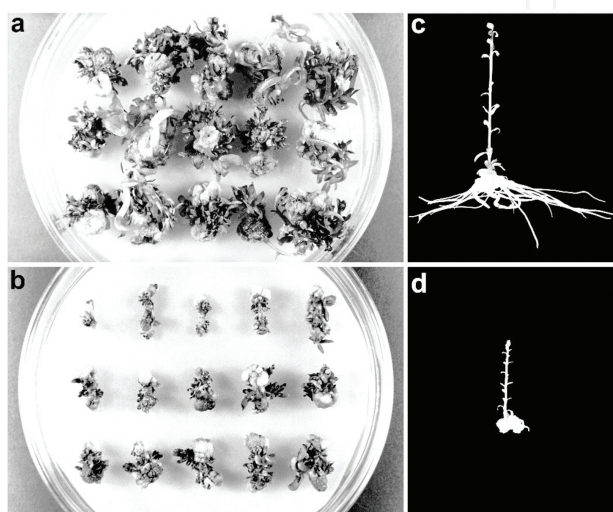


Figure 2. *In vitro* shoot regeneration in water-treated (a) and non-water-treated (b) hypocotyl explants of cv. '1886 Sel.'. *in vitro* root formation and plantlet development of shoots regenerated from water-treated (c) and non-water-treated (d) explants of cv. '1886 Sel.' [3].

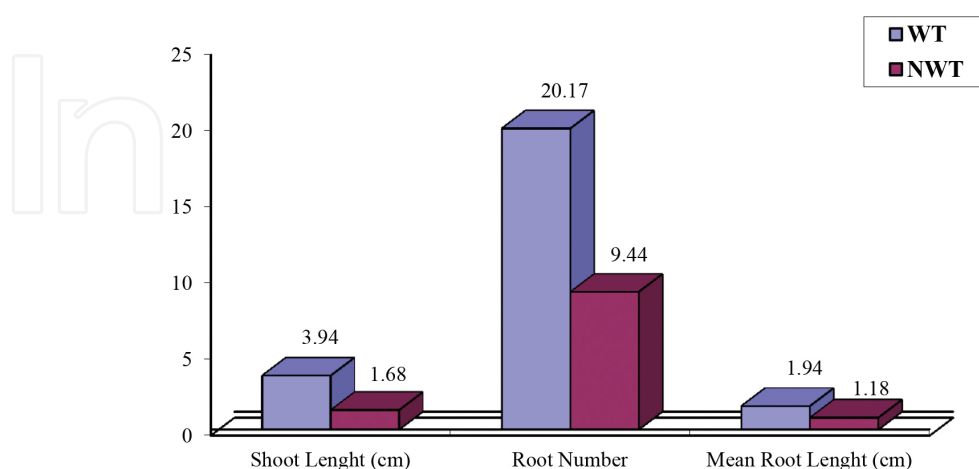


Figure 3. *In vitro* root development of shoots regenerated from water-treated (WT) and non-water-treated (NWT) hypocotyl explants of three flax cultivars ('Madaras', '1186 Sel.' and 'Clarck') on rooting medium enriched with 3 mg l⁻¹ IBA 3 weeks after culture initiation. Value on each the bar is the mean of three cultivars [3].

Shoots got rooted on MS medium supplemented with indole-3-butyric acid (IBA) at a concentration of 3 mg l⁻¹ for 3 weeks. The highest figures were recorded in the shoots regenerated from water-treated tissues (**Figures 2(c)** and **3**).

Statistically significant differences were observed in all parameters between the shoots which were regenerated from water-treated and non-water-treated explants. This sort of effects in water treatment got also noted in the rooting stage. It means that shoots which were regenerated from water-treated explants got more capable of establishing new plantlets than the ones which were grown from non-water-treated explants.

It could be concluded that the lower levels of all parameters of non-water-treated explants were directly due to a decreasing amount of water uptake from the environment and consequently, a reduced mobilization of plant growth regulators. Application of water treatment to explants before culture initiation enriched the tissue's water content and so enabled all solutes and plant growth regulators to transfer into the tissue, providing all cells with a high regeneration capacity and consequently, increasing explant's tissue culture response. Increased growth in water-treated explants was confirmed by Naylor's [10] study which stated that plant growth regulators promote cell division and cell elongation. It has also been reported that decreased germination and seedling growth in stressed rice seedlings was due to decreased mobilization of starch and α -amylase activity [11].

It is understood that pretreatment of explants with water before culture initiation increased the permeability of the epidermis layer and caused to high metabolic activity by increased uptake of water and hormone from the growth medium. Higher fresh and dry weights of water-treated hypocotyls at the end of culture could be attributed to an increase in the absorption of water and other components from the growth medium by means of high permeable epidermis membrane. Water-treated tissues were observed bigger in size than non-water-treated ones in all cultivars as reported by Dale [12], who pointed out that the fresh weight increase causes the cell enlargement with water absorption, cell vacuolation and turgor-driven wall expansion in this study. The increase in dry weight got closely related to cell division and new material synthesis [13]. Dry weight increase of water-treated tissues is caused by an increase in carbohydrate metabolism resulting from the increased water uptake. Besides, lower levels of all the parameters of non-water-treated tissues caused directly a decreased water uptake through the environment and nevertheless, a decreased mobilization of plant growth regulators. Inhibition of the cell division, elongation of cell, or both of them led to the inhibition of growth under water stress conditions [14]. Cell elongation is affected by osmotic water absorption. Osmotic stress lead to biochemical changes in cell wall during growth [15]. Osmotic stress inhibits water uptake which is vital for germination and growth [16]. And water stress affects the level of plant hormones significantly [17].

3. The effect of increased water absorption on shoot regeneration

In another study conducted by Yildiz et al. [4], hypocotyl explants of three flax cultivars ('Omega', 'Fakel' and 'Ariane'), which were pretreated and non-pretreated before culture, were cultured for regeneration. In the study, two regeneration methods, which were based on two

different pretreatment applications, were compared with the conventional regeneration protocol in which explants were directly cultured on MS medium supplemented with 1 mg l^{-1} BAP and 0.02 mg l^{-1} NAA. Hypocotyl explants were kept in sterile cabin under air flow for 30 min in order to make them dry as reported by Christmann et al. [18] in the first and second pretreatment applications in order to decrease the tissue water content and to help the tissues gain the ability to uptake increased amount of water, all solutes and plant growth regulators from the growth medium via tissue's higher osmotic pressure. Later, explants were submerged in MS solution having 1 mg l^{-1} BAP and 0.02 mg l^{-1} NAA for 15 min in both pretreatment applications. Then, explants were cultured on MS medium without growth regulators in the first pretreatment application and on MS medium containing 1 mg l^{-1} BAP and 0.02 mg l^{-1} NAA in the second pretreatment application. It was thought that drying of explants under air flow in sterile cabin increased tissue's osmotic pressure and enabled all cells to absorb more growth regulators along with water in both pretreatment applications by immersing explants into liquid. On the other hand, explants were cultured on MS medium containing 1 mg l^{-1} BAP and 0.02 mg l^{-1} NAA only in the second pretreatment application that means tissues maintained uptaking water and growth regulators from the medium and this led to the higher results in all parameters studied as noted by Yildiz and Ozgen [3]. Okubo et al. [19] has reported that regeneration capacity was affected by endogenous hormone levels of tissue significantly. Fatima et al. [20] has also reported that plant growth is affected by the internal factors such as chemicals and mineral nutrients. Endogenous levels of growth regulators of the plant tissue determine the amount of exogenous plant growth regulators required for regeneration [20]. It was firstly reported that keeping the explants in sterile distilled water for a while before culture initiation promoted the regeneration capacity of explants by increasing tissue's water content and enabling water, all solutes and growth regulators to transfer into the tissue more easily [3].

In accordance with the results, there were statistically important differences among pretreated and non-pretreated hypocotyls in all cultivars. The highest results in all parameters studied were recorded from the second pretreatment application. On the other hand, the lowest results were obtained from the first pretreatment application in which explants were cultured on MS medium without growth regulators in all cultivars after submerging them in MS solution having 1 mg l^{-1} BAP and 0.02 mg l^{-1} NAA for 15 min (**Figure 4**).

Higher results in the fresh and dry weights could be attributed to higher metabolic activity caused by an increase in the absorption of water and growth regulators from the growth medium. From the results of the second pretreatment application, it might be easily seen that culturing explants on MS medium having 1 mg l^{-1} BAP and 0.02 mg l^{-1} NAA after submerging them in liquid MS medium having 1 mg l^{-1} BAP and 0.02 mg l^{-1} NAA increased the tissue's growth regulators' level leading to the higher fresh and dry weights. In fact, transferring explants on MS0 medium after treating them with liquid MS that has 1 mg l^{-1} BAP and 0.02 mg l^{-1} NAA for a moment in the first pretreatment application, growth regulators of tissues did not seem to be sufficient for high scores according to fresh and dry weights. Culturing explants directly on MS medium containing 1 mg l^{-1} BAP and 0.02 mg l^{-1} NAA were not enough again in the increasing tissue's growth regulators' content to obtain higher scores in characters examined in the non-pretreatment application. All the explants regener-

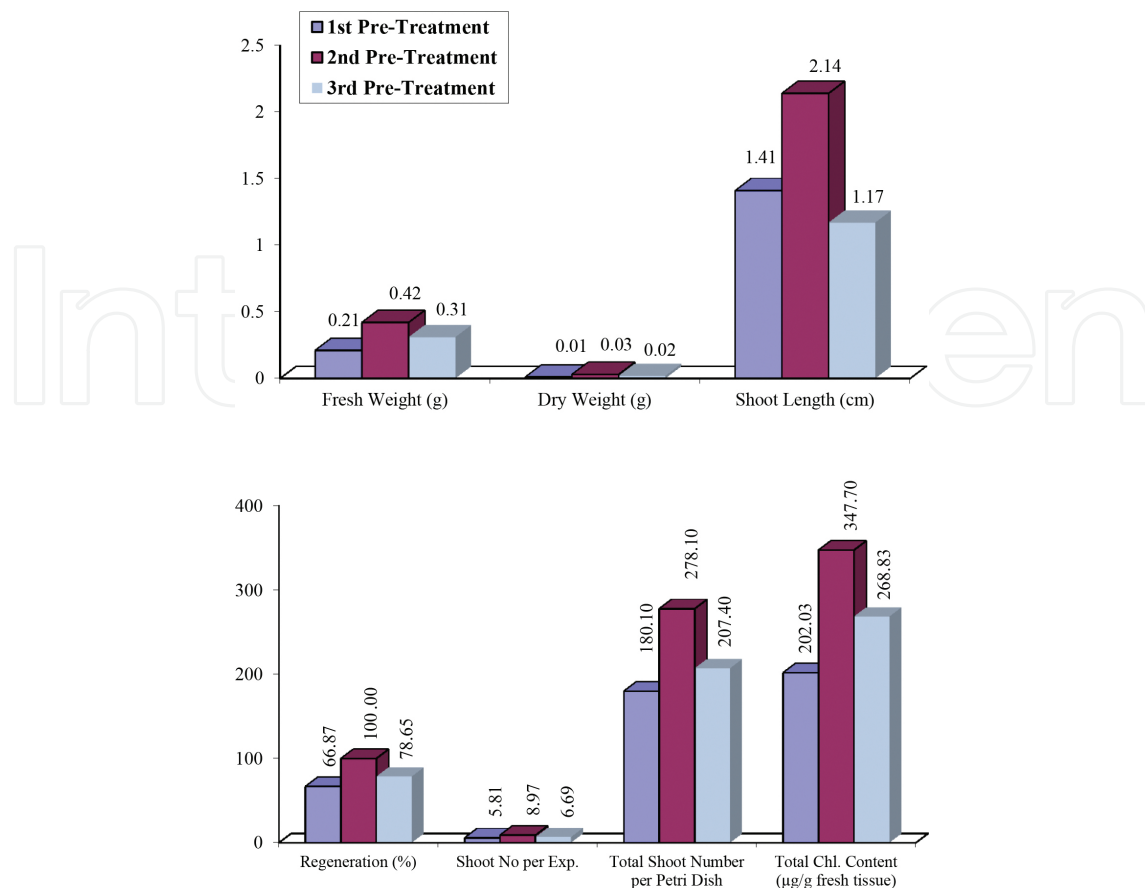


Figure 4. Tissue culture response of pretreated and non-pretreated hypocotyls of three flax cultivars ('Omega', 'Fakel' and 'Ariane') 6 weeks after culture initiation. Value on each the bar is the mean of three cultivars [4].

ated in the second pretreatment application successfully with the regeneration percentage of 100% (**Figures 4 and 5**).

The highest results in shoot number per hypocotyl and shoot length were obtained from second pretreatment application in all cultivars studied. The highest shoot number per hypocotyl was recorded as 8.97. The highest score related to shoot length was 2.14 cm. Shoot regeneration capacity of hypocotyls increased significantly in second pretreatment application. The explants to which second pretreatment application was carried out were more vital and well-grown and more capable of regeneration (**Figures 5(b) and 6(b)**). The highest total shoot number per Petri dish was obtained as 278.10 from second pretreatment application. Total shoot number per Petri dish was reported as a good indicator of the success in both shoot regeneration percentage and shoot number per explant [21]. The highest result of the total chlorophyll content was achieved from the second pretreatment application as 347.70 µg/g fresh tissue. Emerson [22] reported that there exists a close relationship between photosynthesis and chlorophyll content. Chlorophyll content of leaf is thought as a sign of photosynthetic capacity of tissues [22–25] playing a critical role in plant growth and development [26] and its amount alters under stress conditions [27–29]. Gireesh [30] has informed that chlorophyll can be used for measuring growth.

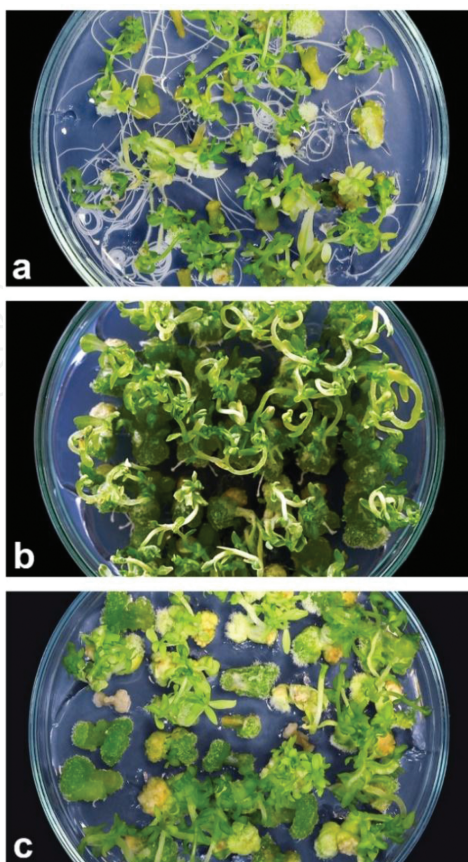


Figure 5. Shoot regeneration from hypocotyl explants of flax cv. 'Omega' [4]. (a) The first pretreatment application: hypocotyls dried for 30 min in sterile cabin and then they were imbibed to liquid MS medium containing 1 mg l^{-1} BAP and 0.02 mg l^{-1} NAA for 15 min, and consequently, cultured on MS medium without growth regulators, (b) the second pretreatment application: hypocotyls got dried by waiting for 30 min in sterile cabin and then were imbibed to liquid MS medium containing 1 mg l^{-1} BAP and 0.02 mg l^{-1} NAA for 15 min, and finally, cultured on MS medium having 1 mg l^{-1} BAP and 0.02 mg l^{-1} NAA and (c) non-pretreatment application: hypocotyl explants got directly cultured on MS medium containing 1 mg l^{-1} BAP and 0.02 mg l^{-1} NAA.

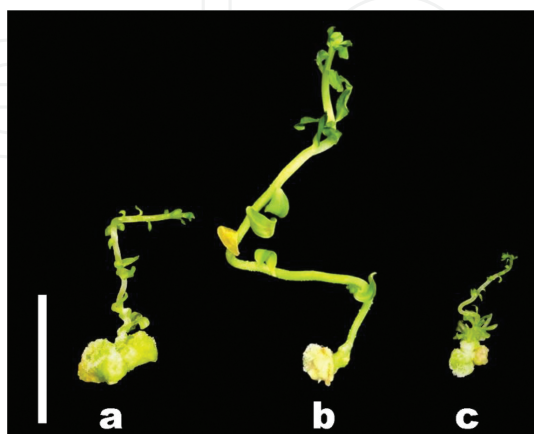


Figure 6. Regenerated shoots of cv. 'Omega' from (a) first pretreatment application, (b) second pretreatment application and (c) non-pretreatment application 6 weeks after culture initiation (bar = 1.0 cm) (original).

In accordance with the results, it might be concluded that the lower levels of all the parameters which were recorded in the first and third pretreatment applications caused from a decreased uptake of water and growth regulators directly from the medium. Higher shoot regeneration has been significantly affected by tissue water content [3]. Keeping explants in liquid medium containing 1 mg l⁻¹ BAP and 0.02 mg l⁻¹ NAA for a while before culture enabled water, all solutes and growth regulators to transfer through the tissue easily, providing all the cells a high regeneration capacity.

4. The effect of water deficiency originated stress in explant on shoot regeneration capacity

In the study conducted by Derelli et al. [5], the effects of water deficiency on shoot regeneration capacity of the explant were evaluated. Flax (*L. usitatissimum* L.) cv. 'Clarck' seeds, which were obtained from 'Northern Crop Science Laboratories', North Dakota, USA, got used in the study. Before germination, seeds were surface sterilized with 40% commercial bleach containing 5% sodium hypochlorite at 10°C for 12 min with continuous stirring and then were washed three to four times with sterile water at the same temperature [31]. Sterilized seeds were germinated on MS medium in Magenta vessels. All cultures were incubated at 24 ± 1°C with a 16-h light/8-h dark photoperiod. Hypocotyl explants were removed as in 5 mm in length from 10-day-old sterile seedlings. Some of the hypocotyls were directly transferred to regeneration medium, while some of them were kept in sterile cabin under air flow for 30 min to decrease the water content of the tissue. Hypocotyl explants were cultured on MS medium which contains 1 mg l⁻¹ BAP and 0.02 mg l⁻¹ NAA. Four weeks after culture initiation, the results obtained from two pretreatment applications were compared with respect to regeneration percentage, shoot number per explant, the highest shoot length per explant and total shoot number per Petri dish.

Regeneration (%)		Shoot number per explant		The highest shoot length (cm)		Total shoot number per Petri dish	
Non-dried	Dried	Non-dried	Dried	Non-dried	Dried	Non-dried	Dried
100	100	4.85	4.10	3.32	2.46	48.50	41.00
t value	0.000 ^{ns}	2.585*		2.296*		2.585*	

^{ns}Not significant.
*Statistically significant at 0.05 level.

Table 1. The effect of water deficiency in explant on tissue culture response of flax (*Linum usitatissimum* L.) cv. 'Clarck'.

The highest results in the study were obtained from the treatment in which hypocotyl explants were directly transferred to regeneration medium without drying. On the other hand, keeping

explants under air flow in sterile cabin for 30 min led to evaporation of more water from the explant and consequently, a decrease in the regeneration capacity (**Table 1**). For this reason, while working in the sterile cabin, explants are to be isolated and placed on growth medium as quickly as possible to protect the regeneration capacity thinking that air flow in the environment can have negative influence on the tissue.

There was no difference between non-dried and dried explants with respect to regeneration percentage. Explants from both treatments formed shoots. The highest results were recorded from non-dried explants, which had higher water content than dried ones, as 4.85, 3.32 cm and 48.50 in shoot number per explant, the highest shoot length and total shoot number per Petri dish, respectively. On the other hand, the lowest results were obtained from dried explants as 4.10, 2.46 cm and 41.00, respectively (**Table 1**).

Lower results from dried explants could be attributed to a decreased water potential of explant tissue and difficulty in distribution of all solutes and growth regulators among cells.

5. Conclusion

The purpose of tissue culture studies is to obtain high-frequency shoot regeneration that is also a prerequisite for an efficient transformation system and a clonal propagation of plants. The introduction of foreign genes which code agronomically important traits into plant cells has not got any meaning if transgenic plants are not recovered from the transformed cell(s). For this reason, tissues with high regeneration capacity should be used. Regeneration capacity of the tissue is the key factor affecting the success of transformation studies. Types, concentrations and combinations of plant growth regulators affect *in vitro* explant growth significantly. Correct concentrations and combinations of auxins and cytokinins should be determined to obtain high frequency adventitious shoot regeneration for related genotype. However, determining the explant type, and the correct concentrations and the combinations of growth regulators is not sufficient for the high frequency shoot regeneration. Shoot regeneration frequency can always be higher than the one we obtain in theory, as every cell has got an ability to form a whole fertile plant under *in vitro* conditions. Many factors affecting regeneration capacity of explant have not been found out yet. Such as, a recently reported technique that utilizes competition among the explants is quite effective to increase shoot regeneration capacity [2]. In this way, the unknown factors affecting regeneration capacity of explants ought to be determined to increase the success of tissue culture studies. In this chapter, the importance of water on shoot regeneration capacity as a main component of all living cells was discussed. Results of research studies given in this chapter showed that enriching tissue with water give rise to higher values with respect to tissue culture response. On the contrary, water deficiency in tissue decreased the regeneration capacity of explant significantly. From now on, water content of the explant should be considered as one of the most important factors such as growth regulators and explant type regarding higher tissue culture response.

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