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# The Prerequisite of the Success in Plant Tissue Culture: High Frequency Shoot Regeneration

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Additional information is available at the end of the chapter

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## 1. Introduction

Plant tissue culture is a term containing techniques used to propagate plants vegetatively by using small parts of living tissues (explants) on artificial growth mediums under sterile conditions. Explants regenerate shoots and roots, and consequently whole fertile plants under certain cultural conditions. Micropropagation is the production of whole plants through tissue culture from small parts such as shoot and root tips, leaf tissues, anthers, nodes, meristems and embryos. Micropropagation is the vegetative (asexual) propagation of plants under *in vitro* conditions and is widely used for commercial purposes worldwide [1-3].

Plant tissue culture techniques have certain advantages over traditional ones of propagation. These are:

- Thousands of mature plants can be produced in a short time that allows fast propagation of new cultivars,
- Endangered species can be cloned safely,
- Large quantities of genetically identical plants can be produced,
- Plant production is possible in the absence of seeds,
- The production of plants having desirable traits such as good flowers, fruits and odor is possible,
- Whole plants can be regenerated from genetically modified plant cells,
- Disease-, pest- and pathogen-free plants in sterile vessels are produced,
- Plants that their seeds have germination and growing problems such as orchids and nepenthes, can be easily produced,

- Providing infection-free plants for mass production is possible.

Plant tissue culture is based on totipotency which means that a whole plant can be regenerated from a single cell on a growth medium. One of the main objectives of tissue culture studies is to obtain high-frequency shoot regeneration, which is also a prerequisite for an efficient transformation system and a clonal propagation of plants with attractive flowers and fruits in large scale for ornamental purposes. Specially the introduction of foreign genes coding agronomically important traits into plant cells has no meaning unless transgenic plants are regenerated from the genetically modified cell(s).

It is known that some families and genera such as *Solanaceae* (*Nicotiana*, *Petunia* and *Datura*), *Cruciferae* (*Brassica* and *Arabidopsis*), *Gesneriaceae* (*Achimenes* and *Streptocarpus*), *Asteraceae* (*Chichorium* and *Chrysanthemum*) and *Liliaceae* (*Lilium* and *Allium*) have a high regeneration capacity while regeneration in some other families such as *Malvaceae* (*Gossypium*) and *Chenopodiaceae* (*Beta*) is difficult. In order to increase the regeneration capacity of explant from the genotype of interest, we have to find answers to such questions as “Why do some genotypes regenerate easily?”, “What can be done to increase the regeneration capacity of explant?”.

## 2. Factors affecting explant's regeneration capacity

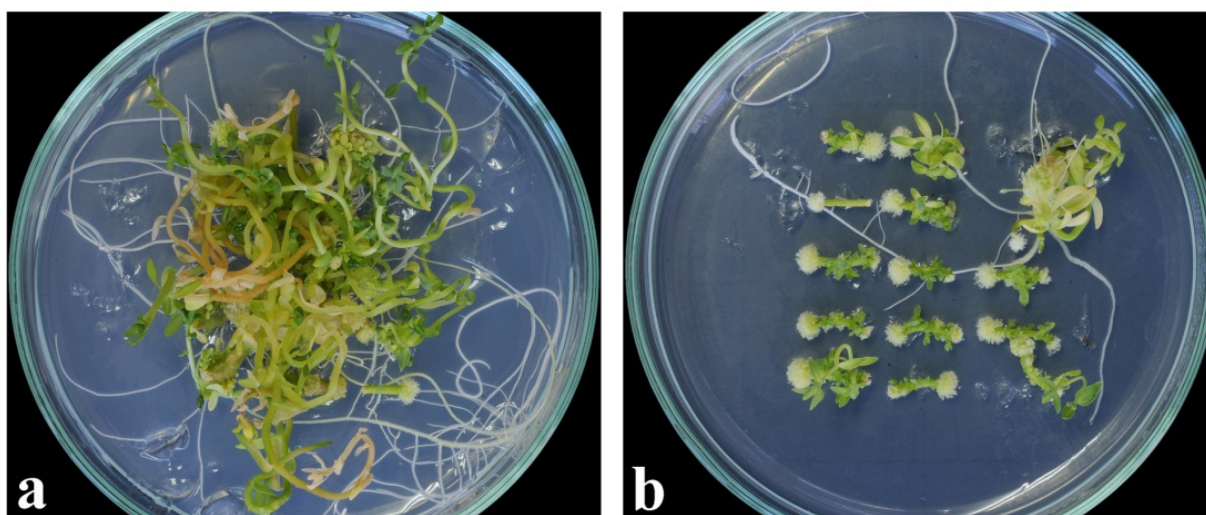
### 2.1. Plant material

Plant material is extremely important for the success of tissue culture studies [4]. Factors affecting explant's tissue culture response are (1) genotype, (2) physiological stage of donor plant, (3) explant source, (4) explant age, (5) explant size, (6) explant position in donor plant and (7) explant density. Plant segments used in tissue culture as explant are stem [5], root [6], leaf [7], flower [8], ovule [9], cotyledon and hypocotyl [10, 11]. Such these explants form direct and indirect organs and embryos. Thin cell layer can also be used as explant in some species [12] while embryos can be successfully used in cereals [13]. Moreover, shoot tips and meristems may give successful results for callus formation and shoot regeneration [14].

#### 2.1.1. Genotype

Regeneration capacity of plants shows a wide range among families, species and even within genotypes from the same species (Figure 1). Generally dicotyledons regenerate more easily than monocots. Plants from some dicotyledon families such as *Solanaceae*, *Cruciferae*, *Gesneriaceae*, *Begoniaceae* and *Crassulaceae* have a high regeneration capacity. In general, herbaceous plants regenerate more easily than woody plants such as trees and shrubs [3].

Sugarbeet from *Chenopodiaceae* family is known as a recalcitrant genotype with respect to *in vitro* culture and genetic transformation [15, 16] (Figure 2) while regeneration and transformation are quite easy in tobacco from *Solanaceae* family [17]. It was reported that somatic embryogenesis changed from 0.00% to 77.50% in 14 maize genotypes cultured *in vitro* [18].



**Figure 1.** Adventitious shoot regeneration from hypocotyl explants of flax (*Linum usitatissimum* L.) cultivars a. '1886 Sel.' and b. 'Clarck' 4 weeks after culture initiation



**Figure 2.** Shoot regeneration from petiole explants of sugar beet (*Beta vulgaris* L.) 4 weeks after culture initiation. Only three explants out of 10 regenerated.

### 2.1.2. Physiological stage of donor plant

Explants show an ability to express totipotency are the most suitable for tissue culture [19]. Generally, vegetative segments of plants regenerate more easily *in vitro* than generative ones [20]. Explants should be isolated from healthy plants with high cell division for successful response to tissue culture. On the other hand, regeneration capacity of mature tissues is quite low. In general, young tissues and organs have a high

regeneration capacity than the older ones. Regeneration capacity of genotypes increases during flowering period as in *Lunaria annua* [21], *Ranunculus sceleratus* [22]. Although plants in a resting stage (dormant) are generally difficult to culture *in vitro* [3], there are some exceptions as flower stem explants of *Tulipa* form shoots only in dry storage (dormant) stage [23].

### 2.1.3. Explant source

Plants grown under greenhouse conditions give rise to better results than the ones grown in field conditions [24]. There are huge variations regarding tissue culture response in explants excised from plants grown in field condition depending on weather conditions during the year [3]. However, the best results are obtained from explants excised from *in vitro* grown seedlings [25].

In a study conducted by Yildiz et al. [25], regeneration capacity of flax (*Linum usitatissimum* L.) hypocotyl explants isolated from *in vitro*- and greenhouse-grown seedlings was compared. Five mm sections of hypocotyl explants from *in vitro*-grown seedlings were directly cultured on MS medium supplemented with 1 mg l<sup>-1</sup> BAP and 0.02 mg l<sup>-1</sup> NAA for shoot regeneration while hypocotyls from greenhouse-grown seedlings were surface-sterilized before culture initiation. The highest values with respect to shoot regeneration percentage, shoot number per explant and total shoot number per petri dish were recorded in explants isolated from *in vitro*-grown seedlings of all cultivars.

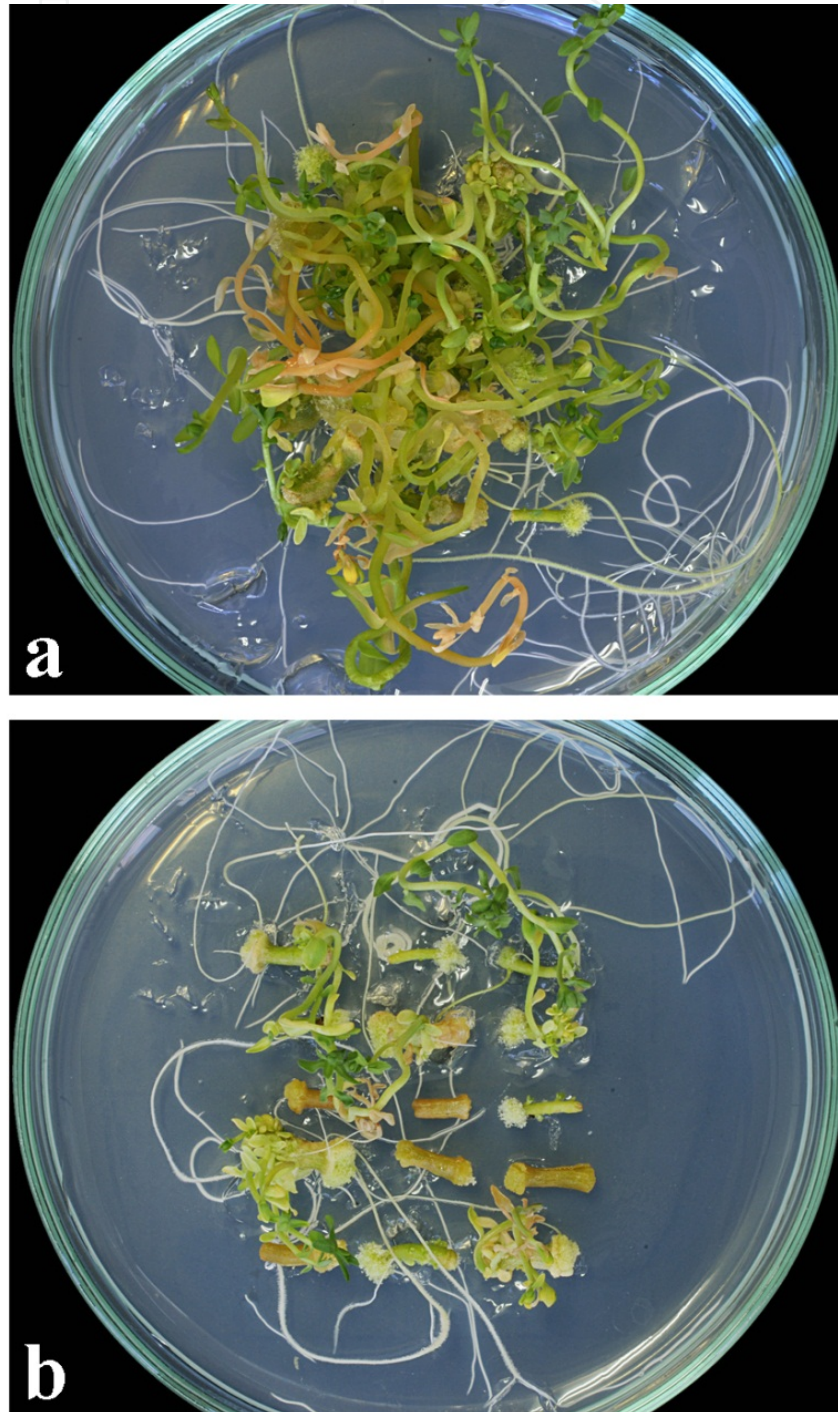
87.50 shoots were formed over 100.00 in explants isolated from *in vitro*-grown seedlings while only 22.50 shoots were recovered in explants of greenhouse-grown seedlings in cv. '1886 Sel.'. The highest shoot number per explant and total shoot number per petri dish were obtained in explants isolated from *in vitro*-grown seedlings as 14.43 and 126.26, respectively. Specially, total shoot number per petri dish was 42.95 times more in explants of *in vitro*-grown seedlings as 126.26 than in explants excised from greenhouse-grown seedlings as 2.94. It was reported that neither shoot regeneration percentage nor shoot number per explant is lonely an indicator of the success of tissue culture studies but 'total shoot number per petri dish' is a good indicator of the success in both parameters for the genotype of interest [26]. These figures in all the parameters indicated the significance of explant source very well.

### 2.1.4. Explant age

Regeneration capacity of older plants is often low. As the organ using for explant source gets older, regeneration capacity decreases. An example of differences in regeneration capacity between young and old seedlings that are used as source of explant was flax (*Linum usitatissimum* L.) [27]. In this study, shoot regeneration capacity of hypocotyl explants excised from *in vitro*-grown seedlings at different ages (7, 12 and 17 days) was examined in three flax cultivars. Explants of 7-day-old seedlings gave rise to the highest results with



respect to shoot regeneration percentage, shoot number per explant and total shoot number per petri dish. The explants of 7-day-old seedlings were reported to be more vital and well grown (Figure 3).



**Figure 3.** Shoot regeneration from flax (*Linum usitatissimum* L.) cv. '1886 Sel.' hypocotyl explants excised from a. 7-day-old seedling and b. 12-day-old seedling 4 weeks after culture initiation

In the study, it was observed that shoot regeneration percentage, shoot number per explant and total shoot number per petri dish varied excessively among explants from different seedling ages. All these three parameters, which were the highest in explants of 7-day-old seedling were reduced significantly in explants of 12- and 17-day-old seedlings. Results clearly showed that there were statistically significant differences in all parameters examined in all cultivars among explants at different ages.

#### 2.1.5. Explant size

It is so difficult to obtain a successful tissue culture response from small parts such as cells and meristems than from larger parts such as leaves and hypocotyls due to their limited nutrients and hormone reserves. Larger explants having a big amount of nutrition reserves such as tubers and bulbs can easily regenerate *in vitro* and are less dependent on nutrients and hormones in growth medium [3].

#### 2.1.6. Explant position in donor plant

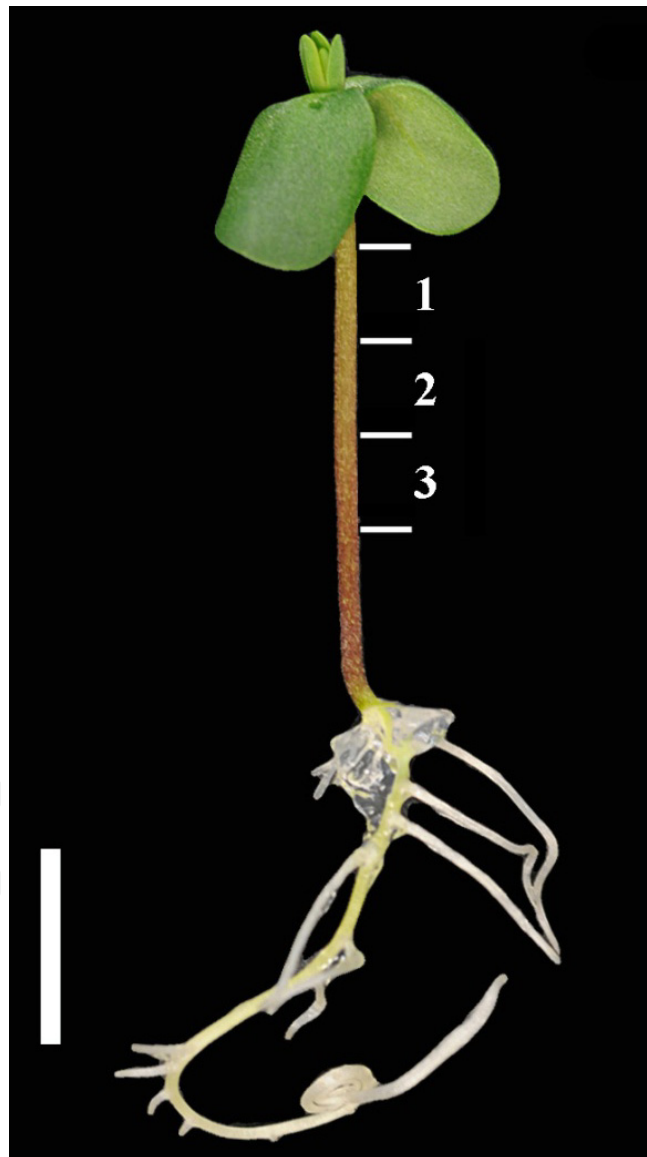
*In vitro* growth of explants can be affected depending on the place from where they are excised. For instance, the higher parts of donor plant are older than the lower parts. Evers [28] has reported that shoot initials from lower parts of *Pseudotsuga menziesii* developed better under *in vitro* conditions. Explants excised from the base produce adventitious bulbs more easily than the top parts of the bulbs [3].

In a study conducted with flax, tissue culture response of hypocotyl explants at different positions was evaluated. Hypocotyls were classified from where they were excised as top, medium and low. Top part was just below the cotyledon leaves while lower one was close to bottom (Figure 4). Fifteen hypocotyls from 7-day-old *in vitro*-grown seedlings were cultured on MS medium 1 mg l<sup>-1</sup> BAP and 0.02 mg l<sup>-1</sup> NAA for 4 weeks.

Explants excised from top (just below cotyledon leaves) part of the seedling gave rise to the highest results with respect to shoot regeneration percentage, shoot number per explant and total shoot number per petri dish. Results were getting lower in the explants excised from medium part of the seedling. And the lowest values were recorded in the explants from lower part (Unpublished study results) (Figure 5).

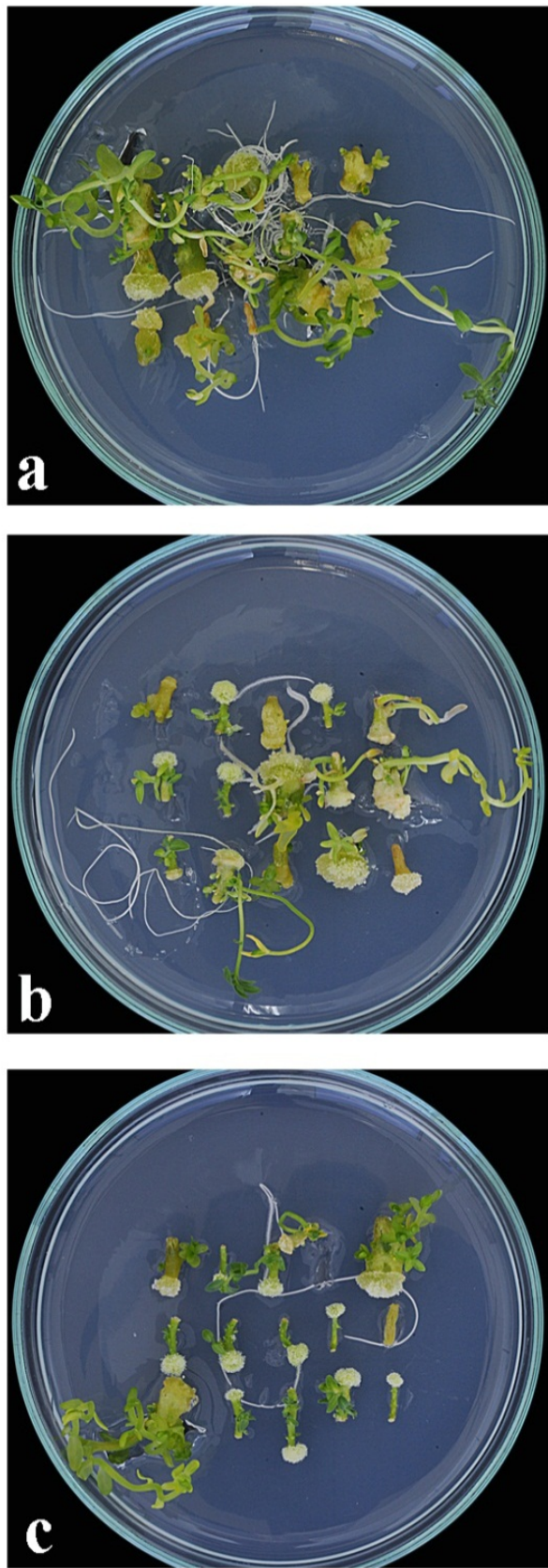
The highest values in shoot regeneration percentage, shoot number per explant and total shoot number per petri dish were recorded in the explants excised from the top of the seedling as 97.78%, 5.17 and 76.00, respectively. These figures were the lowest in hypocotyl segments in the lower part of the seedling as 66.67%, 2.62 and 26.33, respectively. There were huge difference between the results of explants excised from the top and the low parts of the seedling. Shoot regeneration percentage was 97.78% in explants of the top part of the seedling while it was 66.67%. That is 97.78 explants regenerated over 100.00 in the top part of the seedling.

Mean shoot number per explant was recorded as 5.17 in explants excised from the top parts of the seedling while it was 2.62 in explants isolated from the low part of the seedling. Similar results were obtained in total shoot number per petri dish. Shoot number per explant and total shoot number per petri dish are the main indicators of success in plant tissue culture. Especially, after transformation studies via *Agrobacterium tumefaciens*, regeneration capacity of tissue decreases significantly due to plant defense mechanism against pathogen attack. That is why, the number of shoots regenerated should be as much as possible.



**Figure 4.** Seven-day-old flax (*Linum usitatissimum* L.) cv. 'Madaras' seedling and the position from which explants were excised 1. top, 2. medium and 3. low. Bar = 1.0 cm





**Figure 5.** Tissue culture response of hypocotyl explants excised from different positions of flax (*Linum usitatissimum* L.) cv. 'Madaras' seedling a. top, b. medium and c. low

### 2.1.7. Explant Density

There are many studies about competition among the plants in field conditions. Plant leaves compete for irradiance, and roots for water and nutrients [29, 30]. High plant density was accepted as a biotic stress factor [31, 32]. Stoffella and Bryan [33] have reported that plant density has an effect on plant development and yield of many vegetable crops. A linear increase has indicated in fruit yield when plant density is increased [34-36]. Abubaker [37] has noted that the highest planting density gave rise to the lowest yield in bean due to the high competition among plants for water and minerals. Asghari et al. [38] have reported that the chicory plant increased root diameter for increased absorption of water under high density by high competition between plants.

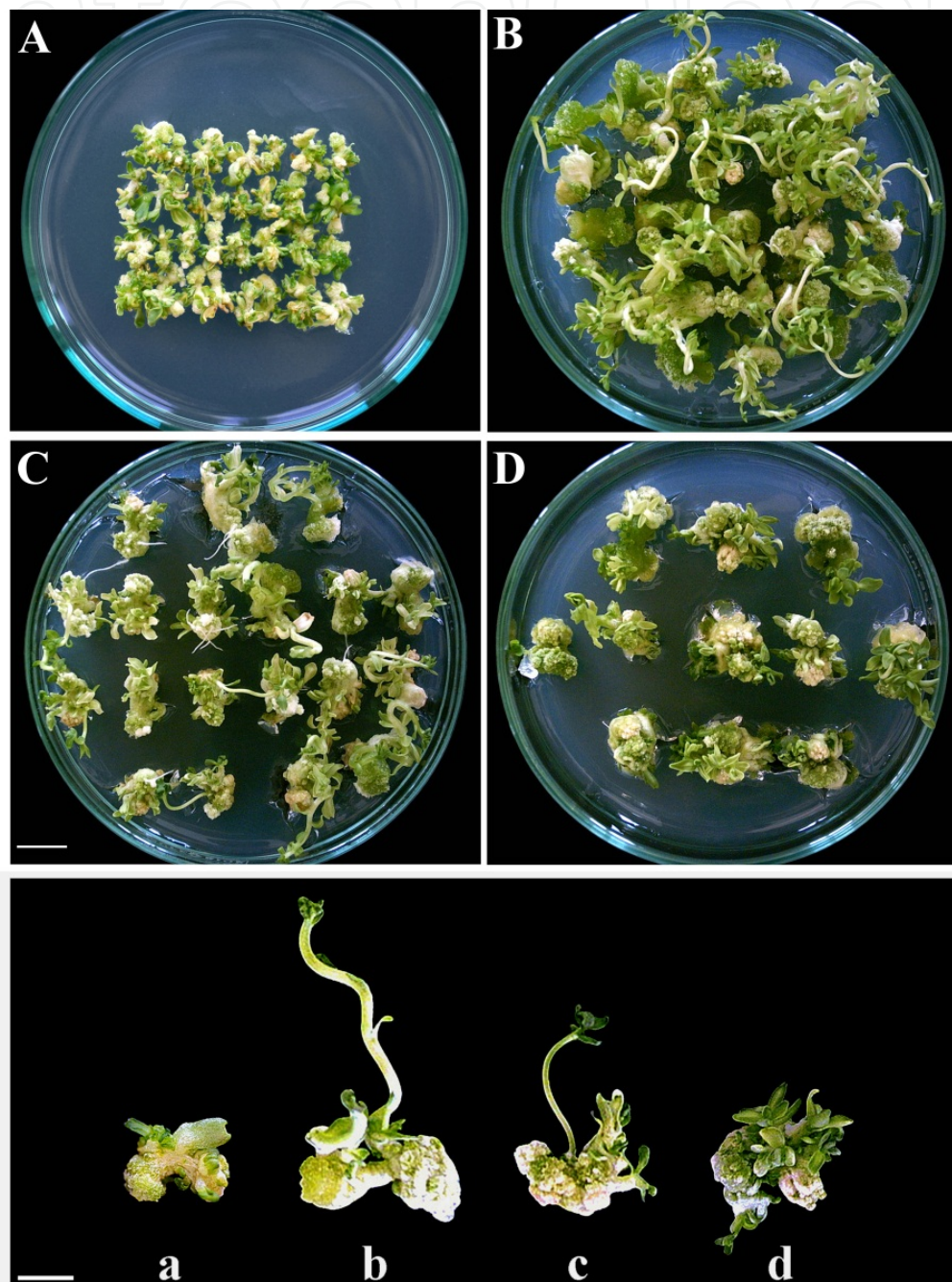
Since a plant is a unity of cells and tissues, behavior of cells and tissues as the smallest unit of the organism, represents the plant's response against any factors arising from nearby environment. Competition and stress are the brother concepts that should be taken into account to increase the success of tissue culture studies. Explants under *in vitro* conditions compete with each other for a constant amount of water and nutrients in growth medium. It was revealed that competition was one of the most important factors increasing explant's regeneration capacity [32].

Yildiz [32] has reported that encouraging hypocotyl explants of flax (*Linum usitatissimum* L.) for competition by decreasing the culture distances among them increased shoot regeneration capacity remarkably till a certain point from where stress initiated and significant decreases in all parameters were observed (Figure 6 and Figure 7). For shoot regeneration, hypocotyls were cultured in a petri dish (90 × 90 mm) at 0.5 × 0.5, 1.0 × 1.0, 1.5 × 1.5 and 2.0 × 2.0 cm distances.

Results clearly showed that there were statistically significant differences in fresh and dry weights in all cultivars among explants cultured at different culture distances. The highest fresh and dry weights per explant of all cultivars were obtained from 2.0 × 2.0 cm distance of hypocotyls and they decreased by decreasing distances. These findings were supported by Gersani et al. [39] and Maina et al. [40] who reported that plants grown alone produce more biomass or yield than those grown with the others. It could be concluded that the decreased distance in which explants were cultured induced stress caused likely by the deficiency of water, sucrose and nutrients. Increases in the fresh and dry weights were chiefly due to an increase in the absorption of water and other components from the basal medium [41]. It was stated that the fresh weight increase was mainly due to cell enlargement by water absorption [42] and increase in dry weight was closely related to cell division and new material synthesis [43].

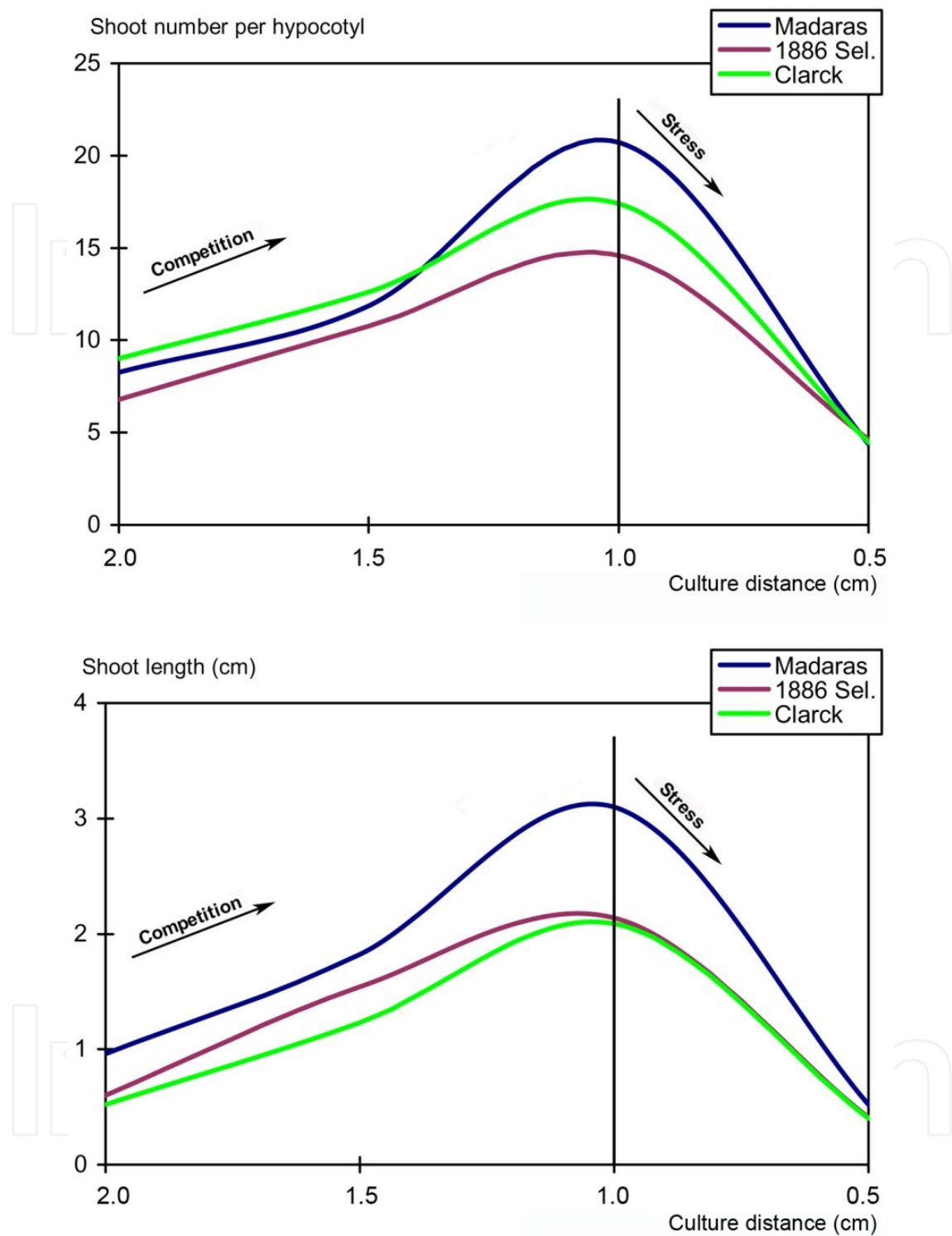
The highest shoot number per hypocotyl (20.70 in 'Madaras', 14.57 in '1886 Sel.' and 17.40 in 'Clarck') and the highest shoot length (3.10, 2.14 and 2.09 cm, respectively) were obtained at 1.0 × 1.0 cm distance in all cultivars studied. It is thought that competition among explants cultured at 1.0 × 1.0 cm distance encouraged them to give higher results than at the other distances.

Plant growth regulators are perhaps the most important components affecting shoot regeneration capacity of explants [44]. In tissue culture studies, correct combinations of auxins and cytokinins have been tried to be determined for high frequency shoot regeneration for the explant [26]. This study has shown that determination of optimum levels of auxins and cytokinins in growth medium is not the only way of increasing shoot regeneration capacity, but also shoot regeneration frequency of explants could be increased simply by encouraging explants into competition.



**Figure 6.** Development of explants cultured at different distances 0.5 x 0.5 cm (A, a), 1.0 x 1.0 cm (B, b), 1.5 x 1.5 cm (C, c) and 2.0 x 2.0 cm (D, d); six weeks after culture initiation. Bar is 1.0 cm for petri dishes and 0.5 cm for shoot regeneration





**Figure 7.** Competition-stress curve of flax cultivars 'Madaras', '1886 Sel.' and 'Clarck' with respect to shoot number per hypocotyl and shoot length

## 2.2. Surface-sterilization process

All tissue culture studies which aim to obtain high-frequency shoot regeneration, which is also a prerequisite for an efficient transformation system, should be performed under sterile



conditions. Explant health is the main factor determining regeneration capacity. Viability of explant and the seedling from which the explant is excised, are very important for high-frequency shoot regeneration [45]. The most important treatment prior to culture initiation is perhaps surface-sterilization of the explant. Since *in vitro* conditions provide bacteria and fungi with an optimal growth environment, unsuccessful sterilization hinders the progress of tissue culture studies. Surface-sterilization process aims to eliminate all microorganisms that can easily grow under *in vitro* conditions; on the other hand, it should guarantee the explant's viability and regeneration capacity, which are known to be affected by the concentration, application period [46] and temperature [45] of disinfectant. Since direct contact of explant with disinfectant during the sterilization process may have a severe effect on regeneration capacity of the tissue [45], using aseptic tissues as source of explant is highly recommended [47, 48].

A wide range of surface disinfectants, such as ethanol, hydrogen peroxide, bromine water, mercuric chloride, silver nitrate, and antibiotics are used for surface-sterilization; however sodium hypochlorite (NaOCl) has been most widely used. NaOCl is highly effective against all kinds of bacteria, fungi, and viruses [49-52]. Moreover, NaOCl has a strong oxidizing property which makes it highly reactive with amino acids [53, 54], nucleic acids [55], amines, and amides [56, 57]. The general reaction between amino acids and NaOCl produces the respective aldehyde,  $\text{NH}_4\text{Cl}$  and  $\text{CO}_2$  [54].

### 2.2.1. Effect of sodium hypochlorite solutions at different concentrations and application periods

*In vitro* seed germination, seedling growth and the viability of the tissue were negatively affected by sodium hypochlorite (NaOCl) at high concentrations [45, 58, 59] while it is ineffective for sterilization of tissues at low concentrations. The negative effects of NaOCl concentration become more severe with increasing application period. Since regeneration capacity of the tissue is negatively affected by higher concentrations and longer application periods of disinfectants [3, 46], sterilization process under *in vitro* conditions should aim to use the lowest concentration of disinfectant for the shortest time.

In the study aiming to evaluate the effects of NaOCl solutions used for sterilization on *in vitro* seed germination and seedling growth in *Lathyrus chrysanthus* Boiss., the best results were obtained from 3.75% NaOCl concentration and 15 min. application period for all parameters examined [60]. Seedborne contamination increased gradually by decreasing concentrations and application periods of NaOCl below 3.75% and 15 min. Dramatic decreases were observed at 5.00% NaOCl concentration in all cases. At this concentration, NaOCl showed deleterious effect on the embryo of the seed. Seed germination decreased to 65.18% when NaOCl concentration increased to 5.00% from 3.75% at 15 min. application period. Seedling growth from seeds sterilized with 3.75% NaOCl concentration for 15 min. were observed growing faster than that of sterilized with other concentrations and application periods of NaOCl (Figure 8). By increasing NaOCl concentration to 5.00%, seedling length decreased to 3.45 cm from 3.90 cm. Higher results in seedlings grown from

seeds sterilized with 3.75% NaOCl concentration for 15 min. could be caused by higher tissue water content as reported that *in vitro* explant growth and plantlet establishment have been affected significantly by tissue water content [41].



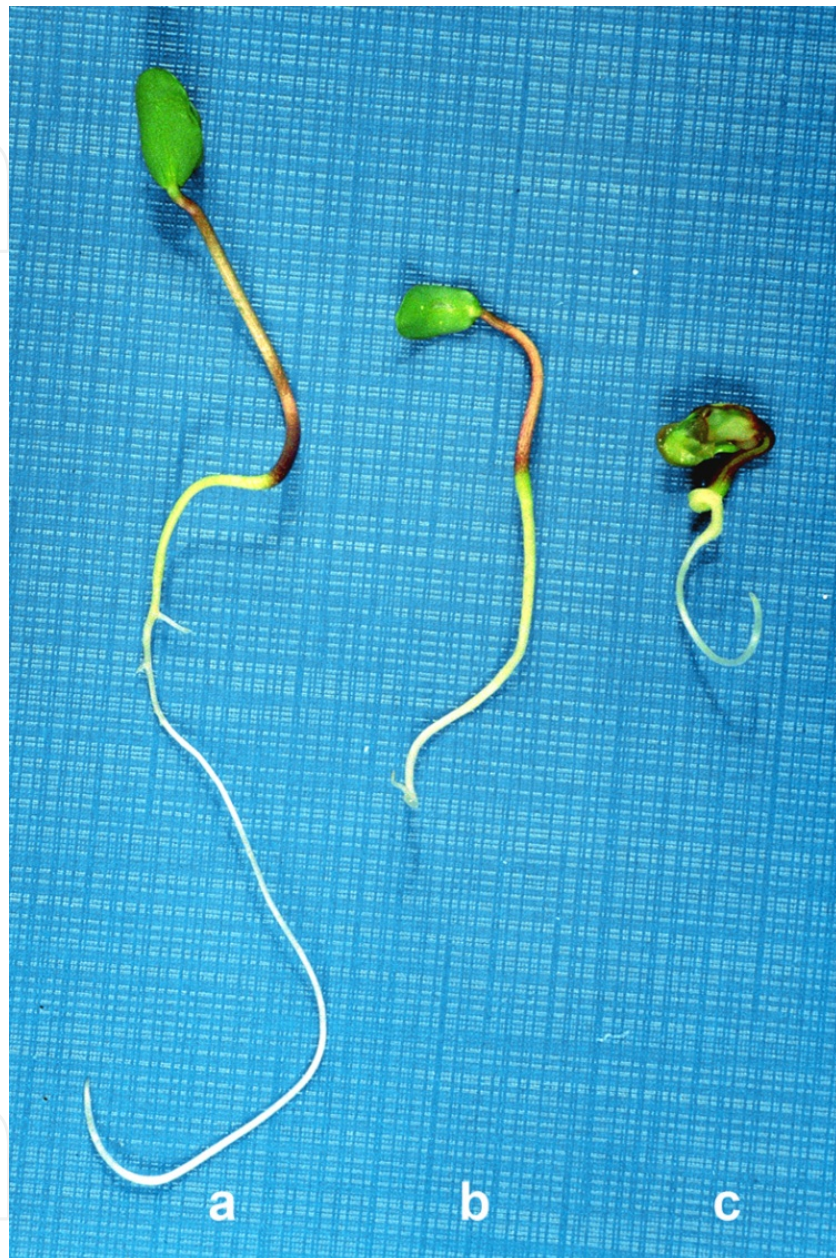
**Figure 8.** *In vitro* seedling growth from *Lathyrus chrysanthus* seeds sterilized with (a) 2.50%, (b) 3.75% and (c) 5.00% NaOCl for 15 min.

### 2.2.2. Effect of sodium hypochlorite solutions at different temperatures

It was firstly reported that besides concentration and application period, temperature of NaOCl was also one of the most important factors affecting *in vitro* seed germination, seedling growth and explant's regeneration capacity [45].

At the 2.00% NaOCl concentration using for surface-sterilization of flax (*Linum usitatissimum* L.) seeds, when the temperature of NaOCl was set below 10°C, bacterial and fungal contamination was observed. However, increases in NaOCl temperature above 10°C resulted in dramatic decreases in seed germination, seedling growth, hypocotyl and root lengths. When flax seeds were surface-sterilized with 3.00% and 4.00% NaOCl concentrations at 30°C, seed germination, seedling growth, hypocotyl and root lengths decreased dramatically. Decreases in all parameters in NaOCl temperature above 10°C could be the fact that disinfection activity of NaOCl increases [61] and disinfectant penetrates more easily through the seed coat [62]. Higher NaOCl temperatures resulted in morphologically abnormal seedlings with stunted hypocotyls and roots (Figure 9).

Yildiz and Er [45] reported that increasing disinfectant temperature using for surface-sterilization of flax seeds to obtain sterile *in vitro* seedlings from which hypocotyls were isolated, reduced shoot regeneration significantly.



**Figure 9.** *In vitro* seedling growth in flax from seeds sterilized with 2.00% NaOCl at temperatures of (a) 10°C, (b) 20°C and (c) 30°C

Regeneration capacity of the explant is negatively affected by higher concentrations, application periods and temperatures of disinfectant used for surface-sterilization. In the sterilization process of the tissues, the concentration, application period and temperature of NaOCl solutions are closely related to each other and they should be considered together. Direct contact of the tissue with disinfectant during the sterilization process may have a severe effect on the viability and regeneration capacity depending on concentration,



temperature and application periods of disinfectant [45, 46]. In addition to this common knowledge, not only seed germination and seedling growth are directly affected by sterilization process, but also regeneration capacity of explants and health of regenerated shoots are indirectly influenced significantly in plant tissue culture studies. That means NaOCl affects the success of *in vitro* studies, from *in vitro* seed germination and seedling establishment to regeneration capacity of the tissue and recovery of plantlets.

### 2.3. Culture medium

The composition of growth medium is an important factor affecting growth and morphogenesis of plant tissues. Plant tissue culture medium consists of macronutrients, micronutrients, vitamins, amino acids or other nitrogen supplements, carbon sources, organic supplements, solidifying agents and growth regulators. Murashige and Skoog [63] are the most commonly used medium in plant tissue culture. The B5 [64], N6 [65] and Nitsch and Nitsch [66] (NN) have been widely used for many plant species. Moreover, for culture of woody species, the Driver/Kuniyuki walnut medium (DKW) [67] and the WPM medium [68] are used. The growth medium is selected for the purpose of tissue culture and for the plant species [69].

Yildiz et al. [27] have conducted a study to evaluate the effects of two different growth medium (MS and B5) and two gelling agents (Agar and Phytigel) on the regeneration capacity of flax (*Linum usitatissimum* L.) hypocotyl explants of three cultivars namely 'Madaras', '1886 Sel.' and 'Clarck'. Results showed that MS (Murashige and Skoog) growth medium and Agar as gelling agent gave rise to the highest results with respect to shoot regeneration percentage, shoot number per explant and total shoot number per petri dish in all cultivars studied (Table 1).

Cultivars	Shoot regeneration (%)				Shoot number per explant				Total shoot number per petri dish			
	1	2	3	4	1	2	3	4	1	2	3	4
'Madaras'	97.22	91.67	80.56	91.67	7.41a	4.70b	2.56cd	2.50cd	86.67a	56.34bc	30.67cde	30.00cde
'1886 Sel.'	100	100	100	100	6.72a	5.56ab	4.14cd	3.95cd	80.34a	66.67ab	49.67cd	47.34cd
'Clarck'	97.22	88.89	88.89	94.44	4.81a	3.02b	2.59bc	2.34bc	57.67a	35.00b	31.00bc	28.00bcd

1. MS medium and Agar; 2. MS medium and Phytigel; 3. B5 medium and Agar; 4. B5 medium and Phytigel  
Values in a row for each cultivar followed by the different letters are significantly different at the 0.01 level

**Table 1.** The effect of different growth medium and gelling agents on shoot regeneration capacity of flax hypocotyl explants

### 2.4. Culture conditions

After explants placed on growth medium for different purposes, they should be cultured in culture rooms where the environmental factors such as temperature and light are controlled. Different species may need different environmental conditions for successful culture.

Lighting in culture rooms is realized by fluorescent tubes. Control equipments of tubes should be set up outside the culture room. Otherwise they may cause over heating inside



the room and in that case extra cooling is necessary. Due to light sources inside the culture room, there should be an efficient cooling system to maintain constant temperature conditions. Fluorescent tubes can be installed under the shelves, above the cultures which provide a more uniform irradiation for the cultures. Although 16h light and 8h dark photoperiod is usually used, there may be some differences according to species grown under long-day or short-day conditions.

The temperature in culture room is so important for successful tissue culture. Temperature variation in culture room should be as small as possible and generally  $\pm 1^{\circ}\text{C}$  is allowed. Otherwise, changing temperature regime causes stress in cultures which is one of the main reasons of unsuccess. That is why, working with many culture rooms are recommended instead of working only with one.

### 3. Treatments increasing explant's regeneration capacity

#### 3.1. Increasing tissue water content

Water is the source of life on earth. Life in a large proportion of terrestrial ecosystems is limited by water availability. The water content of an actively growing plant can be as much as 95% of its live weight. A plant requires water as an essential ingredient of photolysis, the photochemical stage of photosynthesis where water is split using light energy. Neither carbon dioxide nor oxygen required for photosynthesis is usable by plant unless it is in solution in water. Therefore, water is the key to plant's survival and growth. Water is also an excellent solvent. The substances (solutes) that become dissolved in water in plants include mineral ions such as potassium ( $\text{K}^+$ ), sugars (glucose and sucrose), and amino acids, main components of proteins.

The reduction in growth, yield and quality by water stress has been well recognized in field conditions [70-71]. Germination and seedling establishment guarantee plant survival and are very important phases of plant life. Germination rate decreases with decreasing external water potential and for each species there is a critical value of water potential below which germination will not occur [72].

Yildiz and Ozgen [41] have reported that tissue water content affected explant's shoot regeneration capacity significantly. In the study, water-treated and non-water treated hypocotyl explants of three flax cultivars were compared with regard to fresh and dry weights, shoot regeneration percentage, shoot number per explant, shoot length and total shoot number per petri dish. Some hypocotyls were submerged in sterile distilled water with a gentle shaking for 20 min before placing on regeneration medium, while others were directly cultured on MS medium supplemented with  $1\text{ mg l}^{-1}$  6-benzylaminopurine (BAP) and  $0.02\text{ mg l}^{-1}$  naphthaleneacetic acid (NAA) for regeneration. Results clearly showed that there were sharp and statistically significant differences in all cultivars between water-treated and non-water-treated explants concerning all characters examined (Table 2, Figure 10).

Possibly, pretreatment of explants with water softened the epidermis layer and increased the permeability which caused to high tissue metabolic activity by increasing water and hormone uptake from the medium. Thus, increase in the fresh and dry weights of water-treated hypocotyl explants at the end of culture were chiefly due to an increase in the absorption of water and other components from the basal medium via high permeable epidermis membrane. In the study, non-water-treated explants were found smaller than water-treated ones in all cultivars. Dale [42] stated that the fresh weight increase is mainly due to cell enlargement by water absorption, cell vacuolation, and turgor-derived wall expansion. The increase in dry weight was closely related to cell division and new material synthesis [43]. Dry weight increase of water-treated explants is due to an increase in carbohydrate metabolism resulting from increased water uptake. On the other hand, lower levels of all parameters of non-water-treated explants were directly due to a decreased water uptake from the environment and consequently, a reduced mobilization of plant growth regulators. Hsiao [73] has reported that the inhibition of growth under water stress conditions is the result of inhibition of cell division, cell elongation or both. Osmotic water absorption affects cell elongation. It has been suggested that osmotic stress modifies the biochemical changes taking place in the cell wall during growth thereby preventing extension [74]. The primary action of osmotic inhibition is retardation of water uptake which is vital for germination and growth [75]. It has been stated that water stress alters the level of plant hormones [76].

Cultivar	Hypocotyl				Shoot regeneration (%)	
	Fresh weight (g)		Dry weight (g)		WT	NWT
	WT	NWT	WT	NWT		
'Madaras'	0.430±0.047	0.216±0.019	0.034±0.002	0.025±0.002	100±0.000	75.00±2.937
'1886 Sel.'	0.343±0.011	0.231±0.013	0.029±0.013	0.021±0.001	100±0.000	73.34±4.051
'Clarck'	0.396±0.013	0.192±0.025	0.031±0.001	0.019±0.002	100±0.000	78.33±2.305

Cultivar	Shoot number per hypocotyl		Shoot length (cm)		Total shoot number per petri dish	
	WT	NWT	WT	NWT	WT	NWT
'Madaras'	12.17±0.210	8.00±0.328	0.56±0.043	0.25±0.018	182.50±3.142	119.88±4.90
'1886 Sel.'	11.20±0.114	8.15±0.367	0.58±0.029	0.22±0.009	167.88±1.712	122.25±5.487
'Clarck'	10.83±0.265	5.26±0.491	0.60±0.043	0.27±0.020	162.50±3.974	78.88±7.392

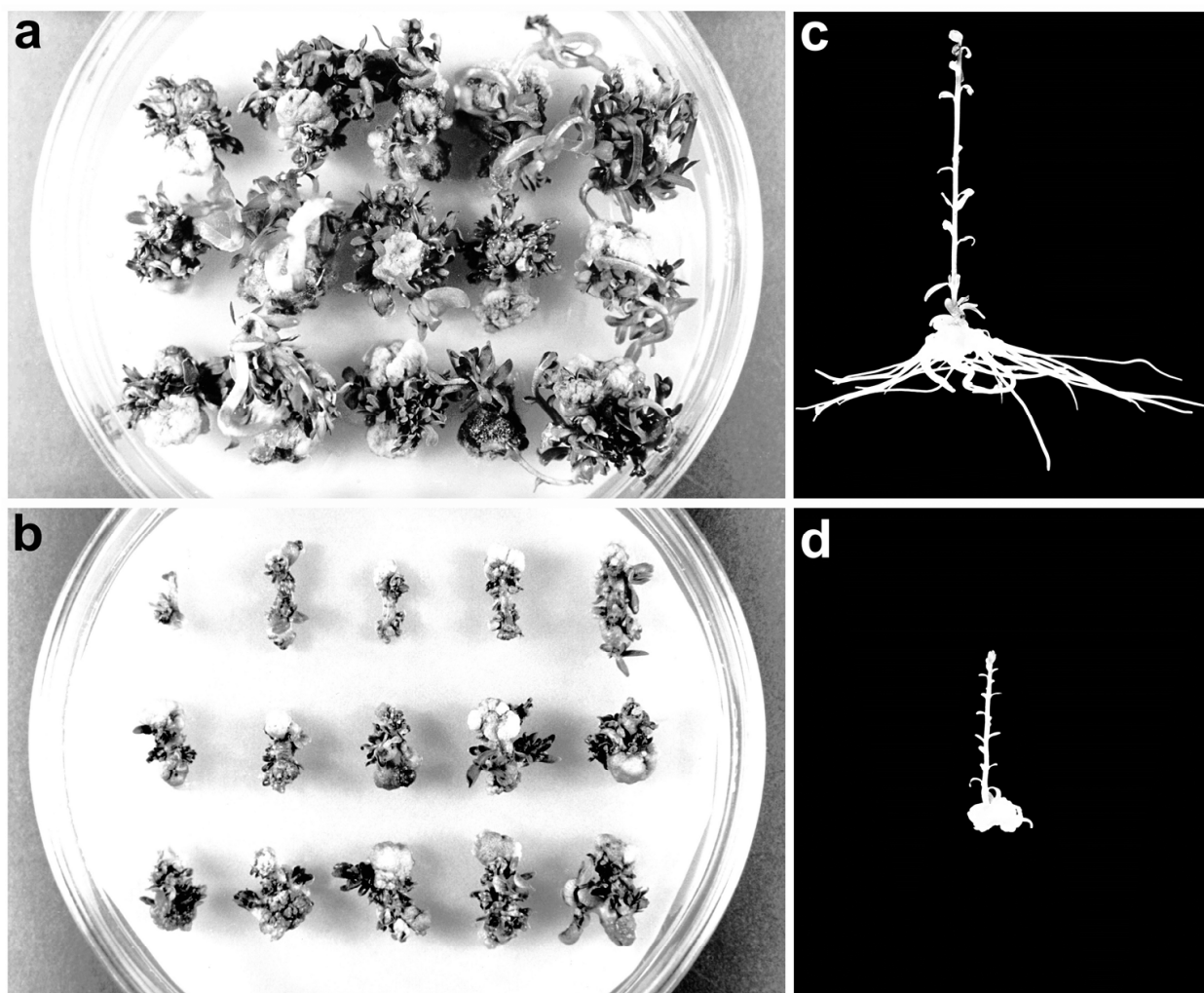
In all cases, the values for WT and NWT were significantly different at 0.01 level with the exception of dry weight of hypocotyls of Madaras which is different at 0.05

**Table 2.** Adventitious shoot regeneration from water-treated (WT) and non-water-treated (NWT) hypocotyls of three flax cultivars 6 weeks after culture initiation on MS medium containing 1 mg l<sup>-1</sup> BAP and 0.02 mg l<sup>-1</sup> NAA.

Treatment of explants with water before culture initiation increased permeability of the epidermis layer and the tissue's water content and so enabled water, all solutes and plant

growth regulators to transfer into the tissue more easily, providing all cells with a high regeneration capacity and consequently increasing explant's tissue culture response.

Shoots regenerated from water-treated and non-water-treated explants were rooted on MS medium containing 3 mg l<sup>-1</sup> IBA for 3 weeks. The best results were obtained in the shoots regenerated from water-treated explants (Figure 10c). Sharp and dramatic differences, which were all statistically significant at the 0.01 level, were observed in all parameters between the shoots regenerated from water-treated and non water-treated explants (Table 3). Similar effects of water treatment were also noted in rooting stage. This means that shoots regenerated from water-treated explants were more capable of establishing new plantlets than the ones grown from non-water-treated explants.



**Figure 10.** *In vitro* shoot regeneration from water-treated (a) and non-water-treated (b) hypocotyls of *Linum usitatissimum* cv. '1886 Sel.' from 6-week-old culture. *In vitro* rooting and development of shoots regenerated from water-treated (c) and non-water-treated (d) hypocotyls of *Linum usitatissimum* cv. '1886 Sel.' 3 weeks later.

Cultivar	Shoot length (cm)		Number of roots		Mean length of each root (cm)	
	WT	NWT	WT	NWT	WT	NWT
'Madaras'	3.02±0.225	1.65±0.209	10.20±1.519	6.50±1.053	1.58±0.156	1.16±0.192
'1886 Sel.'	3.98±0.220	1.28±0.185	21.31±2.121	7.19±1.342	1.92±0.144	0.82±0.076
'Clarck'	4.81±0.396	2.10±0.156	29.00±2.887	14.63±1.812	2.33±0.223	1.56±0.143

In all cases, the values for WT and NWT were significantly different at 0.01 level with the exception of mean length of each root of shoots of Madaras which is different at 0.05

**Table 3.** *In vitro* root development of shoots regenerated from water-treated (WT) and non-water-treated (NWT) hypocotyl explants on rooting medium enriched with 3 mg l<sup>-1</sup> IBA 3 weeks after culture initiation

### 3.2. Regulating osmotic pressure of explant

In another study conducted by Yildiz et al. [77], pretreated and non-pretreated hypocotyl explants of three flax cultivars ('Omega', 'Fakel' and 'Ariane') were cultured for adventitious shoot regeneration. Two different pretreatment applications were compared to the conventional regeneration protocol with respect to hypocotyl fresh and dry weights, shoot regeneration percentage, shoot number per hypocotyl, shoot length and total chlorophyll content. In the 1<sup>st</sup> and 2<sup>nd</sup> pretreatment applications, 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. [78] to decrease the tissue water content and to gain explants the ability of uptaking increased amount of water, all solutes and plant growth regulators by using osmotic pressure in consequent applications. Then explants were treated with MS solution containing 1 mg l<sup>-1</sup> BAP and 0.02 mg l<sup>-1</sup> NAA for 15 min. Finally all explants were cultured on MS medium without growth regulators in the 1<sup>st</sup> pretreatment application and on MS medium enriched with 1 mg l<sup>-1</sup> BAP and 0.02 mg l<sup>-1</sup> NAA in the 2<sup>nd</sup> pretreatment application. It was expected that by immersing explants into liquid regeneration medium after drying enabled all cells to absorb more growth regulators along with water in both pretreatment applications. However, only in 2<sup>nd</sup> pretreatment application, explants were cultured on MS medium containing 1 mg l<sup>-1</sup> BAP and 0.02 mg l<sup>-1</sup> NAA which means that tissues maintained uptaking increased water and growth regulators from the regeneration medium which led to higher results in all parameters studied as reported by Yildiz and Ozgen [41]. Likewise, Okubo et al. [79] have reported that endogenous hormone levels of tissue affected regeneration capacity *in vitro* significantly. Fatima et al. [80] have also noted that internal factors such as chemicals and mineral nutrients affect *in vitro* plant growth. The required amount of exogenous plant growth regulators for cultured tissues depends on the endogenous levels plant tissues have [80]. It was first reported that keeping explants in sterile distilled water for 20 min. before culture on MS medium enriched with 1 mg l<sup>-1</sup> BAP and 0.02 mg l<sup>-1</sup> NAA increased the regeneration capacity of hypocotyls of flax tremendously by increasing permeability of the epidermis layer and tissue's water content and enabling water, all solutes and growth regulators to transfer into the tissue more easily [41].



According to the results, there were statistically significant differences among pretreated and non-pretreated hypocotyls in all cultivars (Table 4).

Cultivar	Pre. App.	Hypocotyl		Shoot regeneration (%) <sup>*</sup>	Shoot number per hypocotyl	Shoot length (cm)	Total chlorophyll content (µg/g fresh tissue)
		Fresh weight (g)	Dry weight (g)				
'Omega'	1	0.25 <sup>**</sup> ±0.020c	0.014 ±0.0019b	82.40±1.07b	6.76±0.46b	1.93±0.13b	217.1±10.40c
	2	0.48±0.023a	0.034 ±0.0023a	100.00±0.00a	11.38 ±0.69a	2.82±0.14a	380.6±26.91a
	3	0.37±0.017b	0.018 ±0.0017b	90.00±5.77ab	7.99±0.74b	1.51±0.17b	286.2±10.45b
'Fakel'	1	0.21±0.027b	0.016 ±0.0030b	72.00 ±7.53b	6.42±0.19c	1.05±0.11b	197.0±15.40c
	2	0.42±0.006a	0.032 ±0.0046a	100.00±0.00a	8.89±0.37a	1.61±0.18a	316.5±14.37a
	3	0.31±0.045b	0.024 ±0.0031ab	80.60±7.45a	7.49±0.10b	0.96±0.10b	252.1±9.89b
'Ariane'	1	0.19±0.024b	0.014 ±0.0023b	46.23 ±6.20c	4.26±0.18b	1.27±0.06b	192.0±11.25c
	2	0.36±0.055a	0.030 ±0.0012a	100.00 ±0.00a	6.64±0.25a	2.00±0.13a	346.0±18.62a
	3	0.26±0.026ab	0.019 ±0.0018b	65.35 ±3.76b	4.59±0.07b	1.06±0.09b	268.2±24.10b

Pretreatment applications;

1. Hypocotyls were waited for 30 min. in sterile cabin under air flow and for 15 min. in solution containing 1 mg l<sup>-1</sup> BAP+0.02 mg l<sup>-1</sup> NAA and finally cultured on MS0 medium

2. Hypocotyls were waited for 30 min. in sterile cabin under air flow and for 15 min. in solution containing 1 mg l<sup>-1</sup> BAP+0.02 mg l<sup>-1</sup> NAA and finally cultured on MS medium containing 1 mg l<sup>-1</sup> BAP+0.02 mg l<sup>-1</sup> NAA

3. Hypocotyls were cultured directly on MS medium containing 1 mg l<sup>-1</sup> BAP+0.02 mg l<sup>-1</sup> NAA (non-pretreated)

<sup>\*</sup>The values represent mean ± standard error of the mean

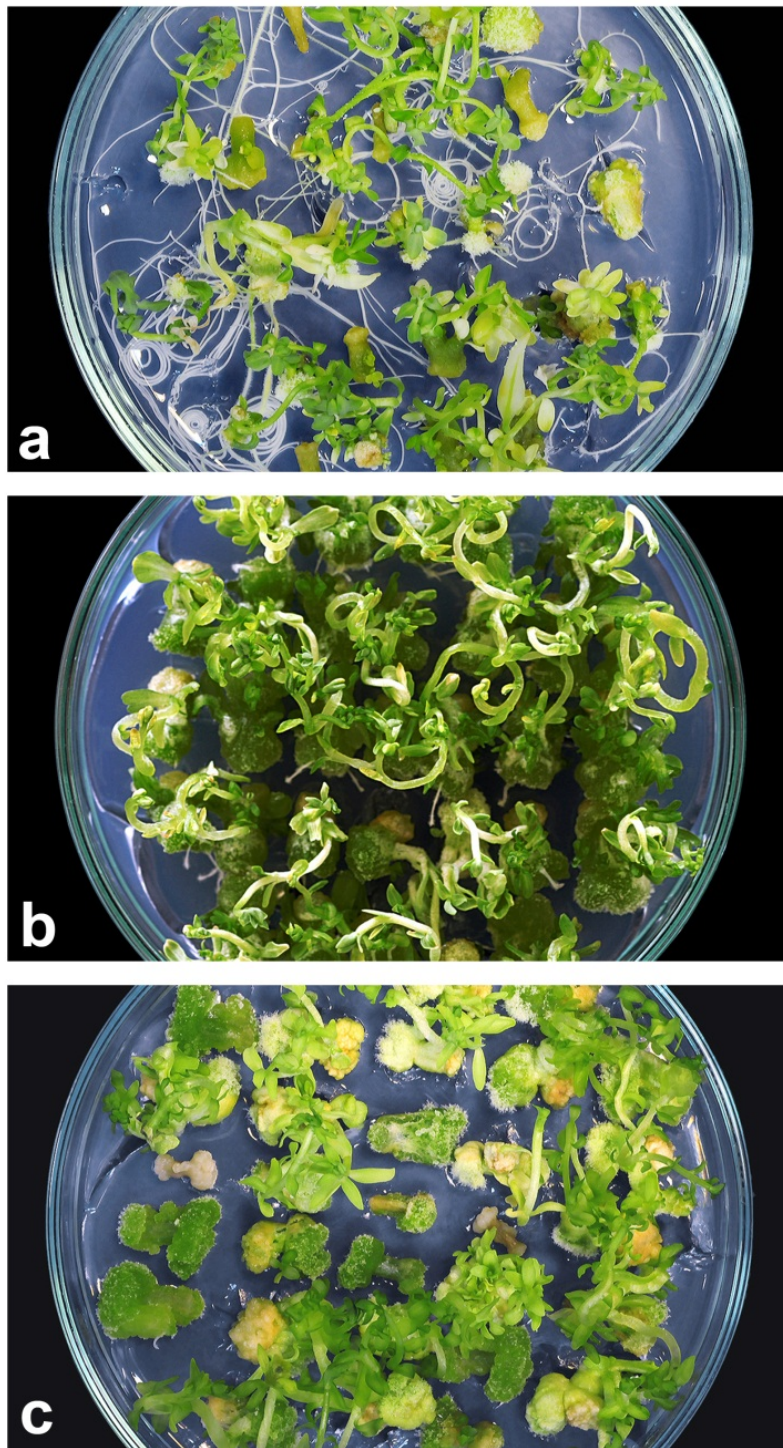
<sup>\*\*</sup>Values within a column for each cultivar followed by different letters are significantly different at the 0.01 level.

**Table 4.** Tissue culture response from pretreated and non-pretreated hypocotyls of three flax cultivars 6 weeks after culture initiation

The highest results in both fresh and dry weights of hypocotyls of all cultivars were obtained from 2<sup>nd</sup> pretreatment application. Scores of fresh and dry weights were followed by non-pretreated hypocotyls. The lowest results were recorded from 1<sup>st</sup> pretreatment application in all cultivars studied (Table 4). From the results, it could be concluded that increases in the fresh and dry weights were chiefly due to an increase in the absorption of water and growth regulators from the medium where explants were first pretreated and then cultured. When the results of 2<sup>nd</sup> pretreatment application were examined, it could be easily seen that culturing explants on MS medium containing 1 mg l<sup>-1</sup> BAP and 0.02 mg l<sup>-1</sup> NAA after treating them with liquid MS medium supplemented with 1 mg l<sup>-1</sup> BAP and 0.02 mg l<sup>-1</sup> NAA clearly enriched the tissue's growth regulators level which caused to higher fresh and dry weights.

The results related to shoot regeneration percentage indicated that the lowest results were obtained from the 1<sup>st</sup> pretreatment application in all cultivars. Hypocotyl explants formed roots and fewer callus in the 1<sup>st</sup> pretreatment application than the others. All explants regenerated successfully in the 2<sup>nd</sup> pretreatment application and consequently shoot regeneration percentage was recorded as 100% in all cultivars studied (Table 4, Figure 11).

The highest results in shoot number per hypocotyl and shoot length were obtained from 2<sup>nd</sup> pretreatment application in all cultivars studied. The highest shoot number per hypocotyl was recorded as 11.38 in 'Omega', 8.89 in 'Fakel' and 6.64 in 'Ariane'. The highest scores related to



**Figure 11.** *In vitro* shoot regeneration from pretreated and non-pretreated hypocotyls of flax (*Linum usitatissimum*) cv. 'Omega'. **(a)** 1<sup>st</sup> pretreatment application: Hypocotyls were waited for 30 min. in sterile cabin under air flow and treated with solution containing 1 mg/l BAP and 0.02 mg/l NAA for 15 min. and finally cultured on MS0 medium, **(b)** 2<sup>nd</sup> pretreatment application: Hypocotyls were waited for 30 min. in sterile cabin under air flow and treated with solution containing 1 mg/l BAP and 0.02 mg/l NAA for 15 min. and finally cultured on MS medium containing 1 mg/l BAP+0.02 mg/l NAA, **(c)** Non-pretreatment application: Hypocotyls were directly cultured on MS medium containing 1 mg/l BAP+0.02 mg/l NAA

shoot length were 2.82, 1.61 and 2.00 cm in 'Omega', 'Fakel' and 'Ariane', respectively. Shoot regeneration capacity of hypocotyls increased significantly in 2<sup>nd</sup> pretreatment application. The best results in total chlorophyll content were obtained from 2<sup>nd</sup> pretreatment application in all cultivars. The highest scores of total chlorophyll content were recorded as 380.6 µg/g fresh tissue in 'Omega', 316.5 µg/g fresh tissue in 'Fakel' and 346.0 in 'Ariane' (Table 4). The explants to which 2<sup>nd</sup> pretreatment application was carried out were more vital and well-grown and more capable of regeneration (Figure 11). Emerson [81] reported that there is a close relationship between photosynthesis and chlorophyll content. Chlorophyll content of leaf is considered as a sign of photosynthetic capacity of tissues [81-84] which plays a critical role in plant growth and development [85] and its amount changes under stress conditions [86-88]. Gireesh [89] has reported that chlorophyll can be used to measure growth.

From the results, it could be concluded that the lower levels of all parameters recorded in the 1<sup>st</sup> and 3<sup>rd</sup> pretreatment applications were directly due to a decreased uptake of water and growth regulators from the medium. Tissue culture response has been affected significantly by tissue water content [41]. Treatment of explants with liquid MS medium containing 1 mg l<sup>-1</sup> BAP and 0.02 mg l<sup>-1</sup> NAA for a while before culture initiation enabled water, all solutes and plant growth regulators to transfer into the tissue much more, providing all cells with a high regeneration capacity and consequently increasing explant's tissue culture response.

#### 4. Conclusion

Plant tissue culture techniques help us to propagate plants vegetatively in a large amount starting from small parts of a tissue and by using the potential of known as totipotency, to form a whole, fertile plant. Plant tissue culture studies are performed on an artificial growth medium under sterile conditions. Explants regenerate shoots and roots, and consequently whole fertile plants under certain cultural conditions. Tissue culture studies aim to obtain high-frequency shoot regeneration, which is also a prerequisite for an efficient transformation system and a clonal propagation of plants. The introduction of foreign genes coding agronomically important traits into plant cells has no meaning unless transgenic plants are regenerated from the genetically modified cell(s). Therefore, using tissues having high regeneration capacity is extremely important. Regeneration capacity of cells or tissues to be used in transformation studies, affects the success of genetic transformation significantly. The types and concentrations of plant growth regulators in plant cell culture significantly affect growth and morphogenesis. In order to obtain high frequency adventitious shoot regeneration for related genotype, correct concentrations and combinations of auxins and cytokinins should be determined. However, determining the explant type, and correct concentrations and combinations of growth regulators is not enough for high frequency shoot regeneration. Since every cell has an ability of forming a whole fertile plant under *in vitro* conditions, shoot regeneration frequency can always be higher than we obtain in theory. Many factors affecting regeneration capacity of explant are not found out yet. For instance, a recently reported technique utilizing competition among explants is very effective to increase shoot regeneration capacity. Thus, unknown factors affecting regeneration capacity of explants should be determined in order to increase the success of tissue culture studies.



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