# IN VITRO PROPAGATION OF A MEDICINAL PLANT

# WITLOOF CHICORY (CICHORIUM INTYBUS L. )

**by**

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## ABSTRACT

## IN VITRO PROPAGATION OF A MEDICINAL PLANT WITLOOF CHICORY (CICHORIUM INTYBUS L.)

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An effcient protocol has been developed for the regeneration of plantlets of witloof chicory, a medicinal plant. After sterilization, chicory seeds were germinated on hormone-free MSMO basal medium (control medium). Leaf petiole and lamina explants were placed onto medium containing various concentrations of KIN and IAA alone or in combinations. Lamina explants cultured on medium containing 0.5 mg/l KIN + 0.3 mg/l IAA had the best response for the shoot formation (19.65 shoots per explant) after 3 weeks cultivation. For later experiments, lamina explants were used due to its higher rate of shoot regenaration capacity. In additon to KIN and IAA, different types of plant growth regulators (NAA, BAP and TDZ) were also tested for shoot formation. In this case, callus formation was remarkable on medium containing 0.3 mg/l NAA in combination with 0.5 mg/l BAP or KIN.

Although TDZ at low concentrations in combination with 0.3 or 1.0 mg/l IAA had particular response for the shoot formation, the leaf morphology was abnormal when the well-developed chicory leaves were considered. For rooting stage, shoots were placed in auxin rich medium containing different types of auxins, (2,4-D, NAA, IAA and BAP). The best root formation per explant was found in medium containing 0.5 mg/l IAA (4.24 roots per explant). Moreover, mean values of root number, shoot and root lengths per explant were reduced at higher concentrations of IAA. For chicory, 2,4-D or NAA was not preferable for root formation, since, during root induction, 2,4-D or NAA even at low concentrations reduced both root and shoot development. The seedlings developed on medium containing IAA or IBA were placed in the pots and transferred to the growth chamber in order to be adapted to non-sterile conditions (acclimatization). Thus, *in vitro* propagation of witloof chicory was completed after 8- 10 weeks from initiation.

**Key words:** *Cichorium intybus* L.*,* medicinal plant, plant growth regulators, *in vitro* culture, callus formation, shoot regeneration, root formation.

# ÖZET

## TIBBI BİR BİTKİ OLAN YABANİ HİNDİBANIN (CICHORIUM INTYBUS L.) IN VITRO ÇOĞALTILMASI

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Tıbbi bir bitki olan yabani hindibanın üretilmesinde çeşitli yaprak eksplantları kullanılmıştır. Öncelikle tohumlar sterilize edilip çimlenme için MSMO hazır besin ortamına alınmıştır. Daha sonra çimlenen bitkilerden yaprak ayası ve sapı, çeşitli hormon içeren besin ortamlarına konulmuştur. Bunlardan yaprak ayası eksplantı, 0.3 mg/l IAA'nın 0.5 mg/l KIN ile kombinasyonunu içeren ortamlarda 3 hafta gelişerek, yüksek sayıda sürgün oluşturmuştur (eksplant başına ortalama 19,65 sürgün).

Deneyin ilerleyen kısımlarında KIN ve IAA'ya ek olarak NAA, BAP ve TDZ kullanılmış ve bunların sürgün oluşumu üzerideki etkisi incelenmiştir.

0.3 mg/l NAA'nın 0.5 mg/l BAP veya KIN ile kombinasyonunda kallus oluşumu göze çarpmıştır. Ayrıca TDZ'nin düşük konsantrasyonlarında yüksek sayıda sürgün oluşumu gözlenmesine rağmen yaprak iyi gelişmemiştir.

Köklendirme için sürgünler oksin (2,4-D, IBA, NAA veya IAA) içeren besin ortamlarına alınmıştır. Bunlardan en iyi sonuç veren 0.5 mg/l IAA içeren ortam olup (sürgün başına ortalama 4,24 kök) IAA'nın yüksek konsantrasyonlarında bu sayı düşmüş ve ayrıca sürgün ve kök gelişiminin yavaşladığı görülmüştür. Bunların dışında köklendirme için kullanılan 2,4-D veya NAA kök ve sürgün gelişimi açısından düşük sonuçlar vermiştir.

İklimledirme için IAA veya IBA içeren ortamlarda gelişmiş sürgünler saksılara alınmış ve dış ortama uyum sağlama süreci başlatılmıştır. Böylece, yabani hindiba üretimi 8-10 haftada tamamlanmıştır.

**Anahtar kelimeler:** *Cichorium intybus* L., tibbi bitki, bitki büyüme düzenleyicileri, *in vitro* kültür, kallus oluşumu, sürgün üretimi, kök oluşumu.

To Sibel

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## LIST OF ABBREVIATIONS

| Abbreviations | Full Name |
|---|---|
| 2,4-D | 2,4-Dichlorophenoxyacetic acid |
| ANOVA | Analysis of variance |
| BAP | Benzylaminopurine |
| EtOH | Ethanol |
| IAA | Indole-3-acetic acid |
| IBA | Indole-3-butyric acid |
| KIN | Kinetin |
| MS | Murashige and Skoog' s medium (1962) |
| MSMO | Murashige and Skoog' s minimal organics medium |
| NAA | Naphthaleneacetic acid |
| PGR | Plant growth regulator |
| TDZ | Thidiazuron |

# 1. INTRODUCTION AND LITERATURE REVIEW

## 1.1. Introduction

Plant tissue culture may be defined as a process whereby small pieces of living tissues explants are isolated from plant organism and grown aseptically on a nutrient medium under controlled conditions. Therefore, plant tissue cultures have many applications in biological sciences. Two concepts, totipotency and plasticity, are important for understanding plant cell and regeneration. Totipotency is the genetic potential of a plant cell to produce an entire plant and the ability of a plant cell to perform all the functions of development that are characteristic of zygote. Plasticity is the ability of the plants to adapt to environmental conditions by altering their metabolism, growth and development to suit their environment. When plant cell and tissues are cultured *in vitro* they generally exhibit a very high degree of plasticity which allows one type of tissue or organ to be initiated from another type**.** The tremendous effects of plant tissue culture are not limited to biological researches but also in commerce. To some extent, tissue culture offers numerous significant benefits over traditional propagation methods such as much faster time, independent growth of the plants, large numbers of genetically identical elite clones and disease-free propagules. Since the last century with the discovery of the DNA and genetic scenarios of the life trends, the new epoch for the future prospects of both humankind and nature have been underlined by biological sciences in response to an increase in human population against the decline in food sources simultanously. In our study, we aimed at producing much more cichory in a very short growth period than naturally grown chicory so that chicory production might be speeded up for its value-added products.

**Figure 1.** General view of *Chichorium intybus* L.

## 1.2. Botany of *Cichorium intybus* L.

*Cichorium intybus* L. belonging to Asteraceae family is a perennial herb, 80-90 cm in height with a fleshy taproot up to 75 cm in length (Figure 1). The genus *Cichorium* consits of six species with major distribution areas in Europe and Asia (Table 1). Chicory is an extremely common roadside weed. It is also known as common chicory, blue sailor's succory and witloof. It is probably the most easily recognizable plant in the state because of its big blue flowers and roadside habitat. During the hot summer months the flowers only stay open a short time in the morning. As the days cool the flowers stay open nearly all day.

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**Table 1** Distribution areas of chicory species.

| Species name | Geopraphical distribution |
|---|---|
| *C.intybus* L. | Northern&Southern Europe, Siberia, Turkey, Afghanistan, China, N. Zeland, Madagascar, S. Africa, India, Ethiopia, Australia |
| *C.endiva* L. | S. Europe, Turkey, Egypt, Tunisia |
| *C.spinosum* L. | Eastern Mediterranean, Italy |
| *C.glandulosum* Boiss | Syria, Turkey, Armenia, Iran, Iraq |
| *C.bottae* Defl | Yemen |
| *C.calvum* Schultz-Bip.ex Ascherson | Ethiopia, Afghanistan, Pakistan |

## 1.3. Its Use in Folk Medicine

Owing to medicinal use of this crop, there is a lot of interest in consuming its leaf and root extract to cure some ilnesses such as diarrhea, enlargement of the spleen, fever, vomiting and in traditional systems of medicine the plant is used for the treatment of jaundice, gout and rheumatism. It is also reported that *C.intybus* is a potent anti-hepatotoxic plant (Zafar and Ali, 1998). Besides the examination of liver sections under estimation of anti-hepatotoxicity of this crop, the alcoholic extract of *C. intybus* is also used against pyrorrhea or gingival inflammation (Patel and Bhatt, 1985; Çubukçu et al., 2003 ) and using the aerial part and root extract of chicory, Petrovic et al. (2004) studied the anti-bacterial activity of *C. intybus* to establish the inhibition of growth of tested bacteria that are *Agrobacterium radiobacter s*sp. *tumafaciens, Erwinia carotovora, Pseudomonas fluorescens* and *Pseudomonas aeruginasa.*

Phytochemicals or plant constituents are distrubuted throught the whole chicory plant, but the main contents are present in the root. Bitter substances are lactucin and lactucopicrin belonging to sesquiterpene lactones are found in roots and head of chicory. It has been also reported that the leaves and the roots of the crop contain traces of bitter sesquiterpene lactones such as guaianolides, lactupin, 8-deoxylactupin, eudesmonolides and guanomanolides (De Kraker et al., 1998)

Also esculetin, esculin, cichoriin, umbeliferone and scopoletin are also various substances extracted from chicory (Rehman et al., 2003). Another important metabolite, inulin (a $\beta$-(2-1)-fructose polymer with terminal glucose) is stored in chicory taproot as a reserve carbohydrate.

## 1.4. Economic Importance of the Crop

The chicory is a popular crop in many developed countries, provides a variety of edible products from both leaves and storage roots. The roots which, when roasted become coffee substitute (Table 2), therefore, the degree of success for marketing of witloof chicory becomes significant enterprise. Several patents and invention details of the crop have been defined, one of those patents has been granted for a novel cosmetic composition containing an extract of the aerial parts of *C. intybus* L., which prevents aging and has the capacity to inhibit radical reactions, in particular by chelation of iron (Diot and Bonne, 1991) and one has been patented for inhibition of salmonella growth. An extract of chicory containing oligosaccharides and another process has been patented for the preparation of a range of inulin products containing carbohydrates with various degrees of polymerisation (Rudi and Lohemar, 1999)

**Table 2.** Requirements for roasted coffee-chicory powder.

| No | Characteristic | Requirements (%) |
|---|---|---|
| 1 | Moisture | 5 |
| 2 | Total ash | 3-7.5 |
| 3 | Acid-insoluble | 0.6 |
| 4 | Water-soluble | 30-50 |
| 5 | Caffeine | 0.6 |

## 1.5. Agricultural Practises

*Cichorium intybus* L. is cultivated in countries such as the UK, Belgium, France, Holland, Germany, South Africa, the USA and India. Since the chicory is a hardy plant they can tolerate extreme temperatures during its vegetative and reproductive phases of growth. For successful seed germination, chicory needs a minimum temperature of 21°C, while for good plant growth it requires a moderate and uniform temperature, with the optimum at 18-24 °C

The seed for stecklings is sown in June in temperate zones of northern India. The stecklings are ready in November for storage. After overwintering for 3-4 months at 3-8 °C, the stecklings become fit for replanting in March-April for the seed crop. In the lighest soils the crop is generally ready for lifting 120-135 days after sowing. To make a good coffee substitute, it is important to slice the roots immediately after lifting so that they contain a high percentage of sugar which is apt to ferment and affect the flavour and taste of chicory.

Fertilizer application in chicory has a high requirement of potash for root and seed crop, irrigation after sowing; thinning and finally pest and disease control of the crop must be available (Arya and Saini, 1978; Bais and Ravishankar, 2001).

## 1.6. Tissue Culture Studies in *Cichorium intybus* L.

Most investigations on *in vitro* culture of *C. intybus* L. have involved root and leaf segment cultures (Table 3). We found an early demonstration on *in vitro* regeneration on *C. intybus* L. as the effects of different hormonal treatments on plantlet regeneration were analyzed by Profuma et al., (1985). They demonstrated that Heller medium supplemented with kinetin was very effective for shoot induction.

Park and Lim, (1999) also established an *in vitro* regeneration system for genotypes, Gulio, Cesare, Augustus, and Silia of *Cichorium intybus* L. var. *sativus*, using leaf and petiole segments as explants. They found that the shoot regeneration rate (80%) in medium containing 1.0 mg/l BAP in combination with 0.1 mg/l IAA was highest among other treatments.

Zafar and Ali (1998) studied anti-hepatotoxic effects of root and callus extracts of chicory, using the aseptically, germinated seedlings and innoculated on Murashige and Skoog (MS, 1962). For initiation of root callus, they used 1mg/l KIN in combination with 1 mg/l IAA and the callus maintained in MS medium supplemented with 6 mg/l IAA and 4 mg/l NAA. Somatic embryogenesis in leaves of chicory was studied by Belletre et al. (1999), addition of glycerol (330 mM) as osmoticum to a synthetic culture medium containing 30 mM sucrose resulted in division of embriyonic cells.

Pieron et al. (1993) optimized the nodule induction in Quorin-Lepoivre medium supplemented with BAP and IBA.

**Table 3.** Some of tissue culture applications on *C. intybus* L.

| Origin of Explant | Basal Medium | Medium Growth Component | Growth Response | References |
|---|---|---|---|---|
| Root | Heller's Medium | KIN 0.05 mg/l or KIN 1mg/l | Shoot organogenesis | Profuma et al., 1985 |
| Leaf | MS | BAP 1 mg/l + IAA 0.1 mg/l | Callus formation and shoot organogenesis | Park and Lim, 1999 |
| Root | MS | KIN 1 mg/l + IAA 1 mg/l | Callogenesis | Zafar and Ali, 1998 |
| Hybrid *C.intybus* leaf | M17 | 330 mM Glycerol | Somatic embryogenesis | Bellettre et al. 1999 |
| Leaf | Quoirin-lepoiure | BAP 1 mg/l + IBA 1 mg/l | Nodule formation | Pieron et al., 1993 |
| Leaf | Semi solid MS | 2 µM IAA + 5µM KIN + 1000 mg/l CH | Shoot organogenesis | Rehman etal., 2003 |

Lastly, Rehman et al. (2003) studied *in vitro* regeneration of chicory from leaf explants and they recorded esculin accumulation in plant tissues at different stages of differentiation *in vitro* cultures and compared with *in vivo*-grown plants. In the presence of casein hydrolyzate (CH; 1000 mg/l) and 0.5 µM IAA in combination with 5 µM KIN induced 5.92 ± 0.25 shoot per explant and also they established that esculin accumulation was higher in *in vitro* plants rather than *in vivo*-grown plants.

Although chicory has a definite agricultural process, there appears some limitations during its harvesting due to many diseases such as *pseudomonas spp*. and *Erwinia spp* have been found to reduce the root quality and it is also reported that the bolting prior to harvesting the roots is one of the main disorder in chicory cultivation caused by high temperatures during root development (Park and Lim, 1999).

Chicory will be increasingly used in coffee blends, as a vegetable and in value-added healthcare products. For that, in future, *in vitro* studies including genetic engineering will enhance the potential of this crop for economic benefits.

# 2. MATERIALS AND METHODS

## 2.1. Plant Material and Disinfection

Seeds of *C. intybus* were picked from university's Gölköy campus in Bolu, Turkey. The seeds were first washed with tap water. Later, they were surface disinfected in 70% EtOH for 30 seconds, rinsed with sterilized distilled (sd-$H_2O$) water, and then dipped into 0.1 % $HgCl_2$ for 3 minutes, after washing with sd- $H_2O$ again, the seeds soaked in 5% Domestos (commercial bleach containing 5% sodium hypochlorite) for 5 minutes. The surface sterilization of the seeds completed rinsing them 4-5 times with sd-$H_2O$.

## 2.2. Preparation of Culture Medium

In the experiments, MSMO basal medium supplemented with 3% sucrose and 0.8% agar (Bacto-agar) was used. The MSMO medium contains some inorganic and organic compounds (Table 4).

**Table 4.** The ingredients of MSMO basal medium (Sigma Aldrich).

| Component | (mg/L) |
|---|---|
| Ammonium nitrate | 1650 |
| Boric acid | 6.2 |
| Calcium chloride anhydrous | 332.2 |
| Cobalt chloride • $6H_2O$ | 0.025 |
| Cupric sulfate • $5H_2O$ | 0.025 |
| $Na_2$-EDTA | 37.26 |
| Ferrous sulfate • $7H_2O$ | 27.8 |
| Magnesium sulfate | 180.7 |
| Manganese sulfate • $H_2O$ | 16.9 |
| Molybdic acid (sodium salt) • $2H_2O$ | 0.25 |
| Potassium iodide | 0.83 |
| Potassium nitrate | 1900 |
| Potassium phosphate monobasic | 170 |
| Zinc sulfate • $7H_2O$ | 8.6 |
| myo-Inositol | 100 |
| Thiamine • HCl | 0.4 |

Two different growth media were used, the *germination medium* and *regeneration medium*.

In the germination medium, the seeds were germinated on medium containing 3% sucrose, 4.43 g/l MSMO in addition of 0.8 % agar before adjusting the pH to 5.7-5.8 and autoclaving all suplemented materials at 121°C/ 1 atm for 25 minutes. The *regeneration medium* contained various concentrations of kinetin, KIN, (0; 0.5; 1.0; 1.5; and 2.0 mg/l, Sigma Aldrich) in combination with IAA (0; 0.3 and 1.0 mg/l Sigma Aldrich). Secondly, 0.5 mg/l KIN was replaced with BAP (0 and 0.5 mg/l) in combination with IAA (0 and 0.5 mg/l) and NAA (0 and 0.5 mg/l). And also, Thidiazuron, TDZ, (0; 0.005; 0.01; 0.02; 0.5; 1.0; 2.0 and 3.0 mg/l Sigma Aldrich) alone or in combination with IAA (0; 0.3 and 1 mg/l) was prepared for shoot induction media.

Seeds placed onto the germination medium ( 5 seeds per 25 ml Jar) was kept in growth chamber at 23°C and 55 - 60% humidity under 16 h light/ 8 h dark regime and growth chamber conditions was stable for all experiments. 0.5 -1 $cm^2$ of leaf or petiole explants were excised from these seedlings.

The shoots approximately 3-4 cm long obtained from the regeneration media were transfered to the rooting media. The MSMO supplemented with IAA (0.5, 1.0, 1.5 and 2.0 mg/l); 2,4-D (0.5, 1.0, 1.5 and 2 mg/l); IBA (0.5, 1.0, 1.5 and 2.0 mg/l), NAA (0.5, 1.0, 1.5 and 2.0 mg/l) was used as the rooting media.

Last stage of the study, hardening off, was carried out after rooting the shooted explants for 4 weeks growth period. For hardening off, comercial pot soil (compost) was used after autoclaving at 121 °C/1 atm for 30 min, then the regenerated seedlings were placed into pots.

Explants forming multiple shoots were counted by using a stereo microscope (Olympus C011) and mean value of the number were estimated as mean number of shoot formation per explant. The degree of the callus formations was indicated as plus (+) sign.

The comparison of the treatments was done with standart error of the mean (SE) using ANOVA test (Microsoft office package program) at 5% probability level.

# 3. RESULTS

## 3.1. Multiple Shoot Formation

Shoot formation is needed for any tissue culture protocol. We therefore, tried to find out the effects of different combinations and concentrations of some plant growth regulators on shoot formation.

### 3.1.1. Effects of KIN in Combination with IAA on Shoot Development from Leaf Explants

Leaf lamina and petiole explants excised from four week old seedlings were cultured on medium containing various combinations and concentrations of KIN and IAA (Table 5). We tried to find out shooting response of the KIN in combination with IAA in MSMO basal medium.

In our experiments, two types of plant growth regulator, kinetin (KIN) and indole acetic acid (IAA) and control group without growth regulators were used in the media. It was found that the presence of the cytokinin (KIN) either alone or combined with an auxin (IAA) in the medium induced shoot formation, therefore, control group and other treatments including IAA only, did not show any shooting response at all.

**Table 5**. Comparision of leaf lamina and petiole explants in terms of shoot formation on medium containing different combinations and concentrations of KIN and IAA after 4 weeks culture. The mean values followed by same letter are not significantly different , $P<0.05$ (Data represents mean values ± SE, n=18, experiment was repeated 4 times).

| Plant Growth Regulators | | | Type of Leaf Explant | | | |
|---|---|---|---|---|---|---|
| | | | Lamina | | Petiole | |
| # | KIN (mg/l) | IAA (mg/l) | % of explants forming shoots | Mean number of shoots/explant | % of explants forming shoots | Mean number of shoots/explant |
| **1** | 0 | 0 | 0 | 0 d | 0 | 0 e |
| **2** | 0.5 | 0 | 44% | 4.7± 0.91 c | 33% | 0.5±0.34 d |
| **3** | 1 | 0 | 89% | 7.78±1.65 bc | 88% | 3.71±1.06 c |
| **4** | 0 | 0.3 | 0 | 0 d | 0% | 0 e |
| **5** | 0.5 | 0.3 | 100% | 19.65±1.32 a | 100% | 5.42± 1.61 b |
| **6** | 1 | 0.3 | 78% | 9.5 ± 2.12 b | 100% | 8.29± 1.17 a |
| **7** | 0 | 1 | 0% | 0 d | 0 | 0 e |
| **8** | 0.5 | 1 | 83% | 12.49± 1.51 b | 50% | 0.83±0.48 d |
| **9** | 1 | 1 | 94% | 13.71± 1.86 b | 71% | 2.57± 0.75 c |
| | **Means** | | **54.2%** | **7.54 ± 1.04** | **49.1%** | **2.37 ± 0.60** |

Shoot induction was achieved with addition of KIN, 1 mg/l KIN alone had a capacity to perform shoot formation almost twice higher than 0.5 mg/l KIN in lamina explants. On the otherhand, 0.5 mg/l KIN in combination with 0.3 mg/l IAA sharply increased the shooting response almost five times higher than that of 0.5 mg/l KIN alone. However, regeneration percentage and mean number of shoots per explants decreased with addition of 1 mg/l KIN (from 19.65 to 9.5). While decreasing the shoot formation, 1 mg/l KIN plus 0.3 mg/l IAA increased rate of shooting on petiole explants (8.29 shoots per explant).

Lastly, higher IAA concentration (1 mg/l IAA) with various KIN concentrations dramatically decreased shoot formation in petiole explants. Decline was seen not only in petiole explants but also in lamina explants. The results showed that 1 mg/l IAA combined with 0.5 and 1 mg/l KIN had no difference in terms of average number of shoots per lamina explant (12.49 and 13.71, respectively). In our experiments, almost all regenerated explants showed callus formation during organogenesis, whilst some explants showed necrosis, which then ended up with complete death.

This part of the work was carried out as a preliminary study for our later experiments in order to check which type of the leaf explant (ie, lamina or petiole) could be used for a high rate of regeneration *in vitro*.

### 3.1.2. Effects of BAP in Combination with NAA on Shoot Development from Leaf Explants

In addition to KIN and IAA used in previous experiments, BAP as a cytokinin and NAA as an auxin were also used for shoot formation. Also, in this experiment only leaf lamina was used as an explant source due to its higher regeneration capacity than that of the petiole explants observed in the previous experiments.

As it was described in the previous experiment (Table 5), treatments including cytokinin showed shoot induction and formation in this experiment (Table 6) and mean number of shoots was counted every week after 9 days culture (Figure 2).

Treatment 3 containing 0.5 mg/l KIN combined with 0.3 mg/l IAA has a much higher capacity for shoot formation than 0.5 mg/l KIN alone (treatment 2), whereas addition of 0.3 mg/l NAA (instead of IAA) dramatically decreased shoot formation (treatment 4). Moreover, 0.3 mg/l NAA increased callus formation and caused the explant to have hairy roots and become vitrified.

**Table 6.** Lamina explants after 4 weeks of growth period was evaluated. ± represents SE, n=9 with 2 replicates and experiment repeated 3 times. The means followed by the same letter are not significantly different, (+) represents degree of callusing, Tukey multiple comparasion test, $P<0.05$, n.s., not significant.

| Treatment no (mg/l) | Mean number of shoots/explant | Shoot regeneration (%) | Callus response |
|---|---|---|---|
| 1. Control | 0 e | 0 | n.s |
| 2. KIN 0.5 | 5.5 ± 1.4 c | 42% | + |
| 3. KIN 0.5 +IAA 0.3 | 18.32±1.16 a | 100% | + |
| 4. KIN 0.5 +NAA 0.3 | 1.39 ± 0.25 d | 55% | +++ |
| 5. BAP 0.5 | 9.5 ± 1.33 b | 86% | + |
| 6. BAP 0.5 +IAA 0.3 | 10.72 ± 1.67 b | 89% | ++ |
| 7. BAP 0.5 +NAA 0.3 | 2.17 ± 0.27 d | 83% | +++ |
| 8. IAA 0.3 | 0 e | 0 | + |
| 9. NAA 0.3 | 0 e | 0 | ++ |

On the otherhand, 0.5 mg/l BAP plus 0.3 mg/l IAA (treatment 6) showed similar regeneration percentages comparing to 0.5 mg/l BAP alone. Addition of 0.3 mg/l NAA (treatment 7) again sharply decreased the mean number of shoots (from 10.72 to 2.17). In our experiment, callus response was also considered. The treatments including 0.3 mg/l NAA increased the callus formation in explants much more than the other treatments excluding NAA (Figure 6).

When compared the shooting response of 0.5 mg/l KIN plus 0.3 mg/l IAA (treatment 3) with 0.5 mg/l BAP plus 0.3 mg/l IAA (treatment 6), treatment 3 was approximately fifty percent more regenerative than treatment 6.

In order to compare statistically the means of the treatments in terms of shoot formation, a two factor ANOVA with replications was carried out using Microsoft office Excell package program. The null hypothesis considered all treatments having equal mean number of shoots were rejected, ($F= 34.06$, $F_{0.05, 210, 5}= 2.26$). The data shows that there is a significant difference on shoot formation between auxins -NAA and IAA- ($F=134.66$) and between cytokinins- BAP and KIN-($F=16.06$) as well as their interaction (cytokinin + auxin) between each other ($F=23.25$) corresponding to critical value ($F_{0.05, (2), 1,140} = 3.91$). Therefore, the presence of IAA and KIN in the media significantly affected the number of shoots.

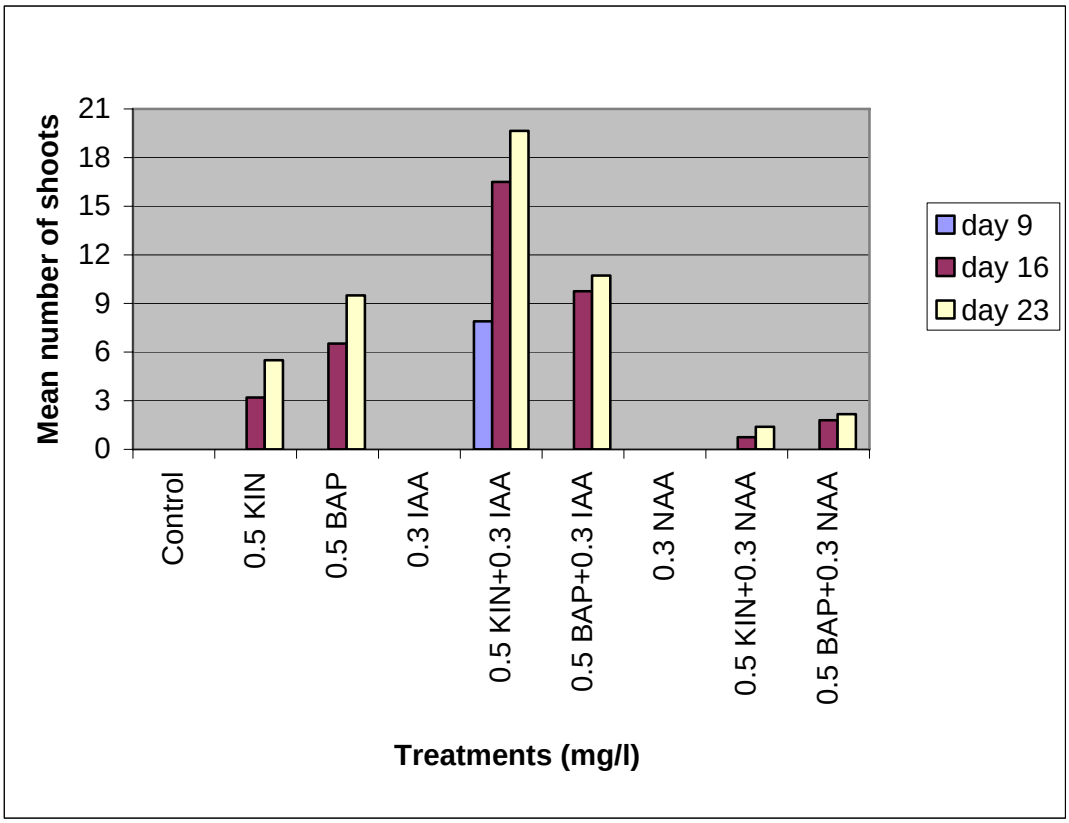
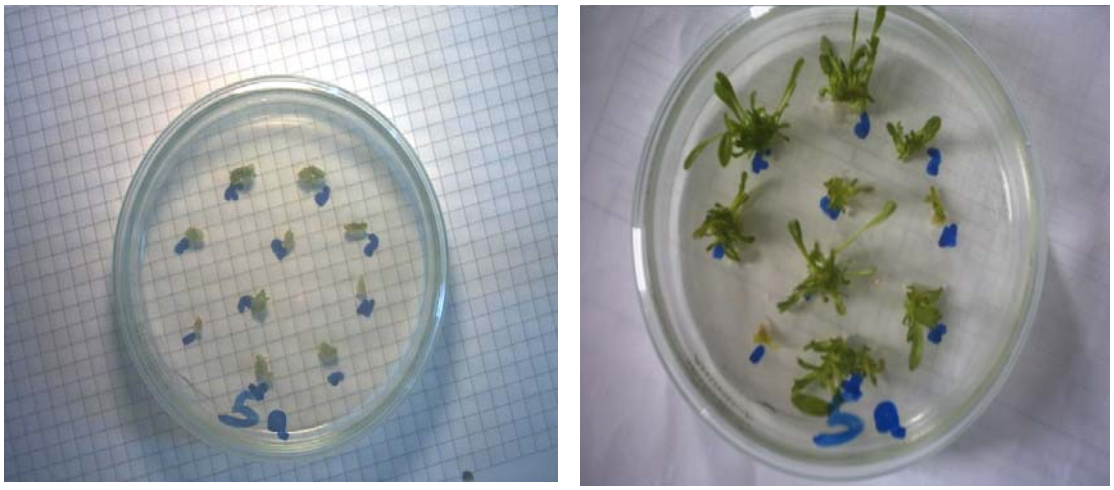



**Figure 2.** Mean number of shoots per explant after 9, 16 and 23 days culture.

Shoot formation of the petiole explants the medium containing 0.5 mg/l KIN plus 3 mg/l IAA is given in (Figure 3), callus becoming specialized in form and function (a) and the component cells of callus have the ability to form a whole plant, (b). Differentiation stage is also clear in a microshoot forming explant in Figure 4.

Shooting response of the treatments varied in auxin and cytokinin ratios was also time dependent, for example 0.5 mg/l BA plus 0.3 mg/l IAA was found in longer period of callus formation phase than 0.5 mg/l KIN plus 0.3 mg/l IAA (Figure 5), therefore, shoot formation of former was late.



**(a)** **(b)**

**Figure 3.** a) Differentiation of the explants. Picture was taken at 9th day of the experiment. b) Explants are determined to induce shoots at17th day

.

**(a)** **(b)**

**Figure 4**. a) Shoot formation of an explant on medium containing 0.5 mg/l KIN at 5th day of the experiment and b) 7th day of the experiment

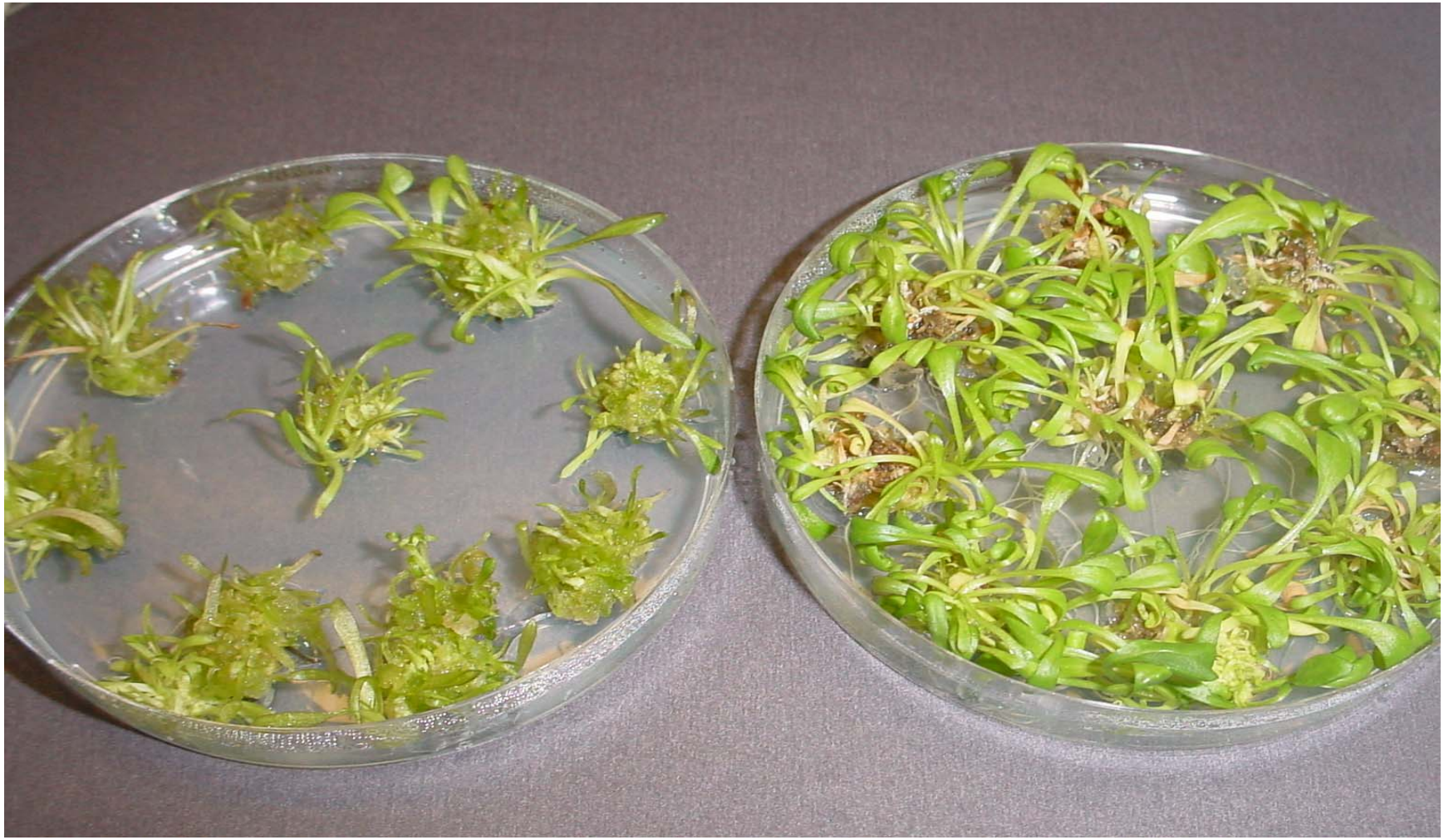


**Figure 5**. Comparision of 0.5 mg/l KIN+ 0.3 mg/l IAA (right) and 0.5 mg/l BAP+0.3 mg/l IAA (left) in terms of shoot formation.

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**Figure 6.** Medium containing 0.3 mg/l NAA performed high amount of callus formation.

### 3.1.3. Effects of TDZ in Combination with IAA on Shoot Development from Leaf Explants

Thidiazuron, a substituted phenylurea (*N*-phenyl- 1, 2, 3 thidiazol 5- ylurea), is a potent bioregulant of *in vitro* morphogenesis. It was reported that TDZ is the most active cytokinin-like growth substances for tissue culture of woody plant species (Ledbetter and Preece, 2004; Singh et al., 2003). Although *C. intybus* was not a woody plant, it was examined by TDZ in combination with IAA for shoot induction.

The data showed that the number of shoots on medium containing 0.005 mg/l TDZ plus 0.3 mg/l IAA increased approximately five times when compared with 0.005 mg/l TDZ alone (Table 7). In this case, gradual increase of TDZ concentration also increases the number of shoots. 0.02 mg/l TDZ alone has shoot formation almost four times higher than that of 0.005 mg/l (from 4.3 to 16). However, 0.02 mg/l TDZ in combination with 0.3 or 1 mg/l IAA increased the shoot number as much as 50 % of the shoot numbers at 0.02 mg/l TDZ alone.

**Table 7.** Mean number of shoots and callus response of the lamina explants on medium containing different TDZ+IAA combinations and concentrations after 4 weeks culture.The mean values followed by the same letter are not significantly different, P<0.05 (Data represents mean values ± SE; (+), degree of callusing, n.s, not significant; n=20, experiment was repeated twice).

| Treatment No | TDZ (mg/l) | IAA (mg/l) | Mean Number of Shoots/Explant | Callus Response |
|---|---|---|---|---|
| **1** | 0 | 0 | 0 f | n.s |
| **2** | 0.005 | 0 | 4.30±0.69 e | + |
| **3** | 0.01 | 0 | 7.85±1.47 d | + |
| **4** | 0.02 | 0 | 16.00±2.31 c | + |
| **5** | 0 | 0.3 | 0 f | n.s |
| **6** | 0.005 | 0.3 | 19.25±2.31c | + |
| **7** | 0.01 | 0.3 | 26.1±2.96 b | + |
| **8** | 0.02 | 0.3 | 31.8±2.11 ab | + |
| **9** | 0 | 1 | 0 f | n.s |
| **10** | 0.005 | 1 | 22.2±2.51 bc | + |
| **11** | 0.01 | 1 | 35.75±2.29 a | + |
| **12** | 0.02 | 1 | 34.45±2.52 a | + |

**Table 8.** Mean number of shoots and callus response of the lamina explants on medium containing higher concentrations of TDZ than the amounts given in Table 5 in combination with 0.3 or 1 mg/l IAA after 4 weeks culture.The mean values followed by the same letter are not significantly different, P<0.05 (Data represents mean values ± SE; (+) degree of callusing, n.s, not significant; n=20, experiment was repeated twice).

| Treatment No | TDZ (mg/l) | IAA (mg/l) | Mean Number of Shoots/Explant | Callus Response |
|---|---|---|---|---|
| **1** | 0.5 | 0.3 | 18.56±1.59 a | ++ |
| **2** | 1 | 0.3 | 6.31±0.58 c | ++ |
| **3** | 2 | 0.3 | 6.06±0.93 c | ++ |
| **4** | 3 | 0.3 | 5.75±0.96 c | ++ |
| **5** | 0.5 | 1 | 10.81±1.96 b | ++ |
| **6** | 1 | 1 | 10.25±1.38 b | ++ |
| **7** | 2 | 1 | 7.69±1.23 bc | ++ |
| **8** | 3 | 1 | 4.94±0.97 c | ++ |

Although 0.02 mg/l TDZ plus 0.3 mg/l IAA has a higher capacity for shoot formation, the leaf morphology was abnormal and seemed not elongated in diameter. In addition to this experiment, higher concentrations of TDZ (0.5, 1.0, 2.0, and 3.0 mg/l ) than used in previous experiment were also examined in combination with 0.3 or 1 mg/l IAA (Table 8).

The shoot formation decreased with increasing TDZ concentrations from 0.5 mg/l to 3 mg/l, where IAA concentration was kept constant at 0.3 mg/l. In this case, the decline in the mean values in treatments 2, 3 and 4 were similar.

Same pattern was also seen in the medium containing 1 mg/ IAA in combination with different concentrations of TDZ ranging from 0.5 to 3 mg/l, however, in this case the mean values of shoot formation for treatments 6 and 7 seem a bit higher than the treatments 2 and 3, most likely due to the presense of 1 mg/l IAA. Considerably, the mean number of shoot formation in treatment 1 involving 0.5 mg/l TDZ plus 0.3 mg/l IAA was higher (approximetly 50%) than that of treatment 5 containing 0.5 mg/l TDZ plus 1 mg/l IAA. 0.5 mg/l TDZ with 0.3 mg/l IAA (treatment 1) has a higher average number of shoots per explant. It was clear that the increase in IAA concentrations also increased the shoot number per explant.

Low and high amount of TDZ in combination with 0.3 or 1 mg/l concentration of IAA directly affected the shoot formation and also callus response of the explant. Callus amount was various between treatments containing lower concentrations and higher concentrations of TDZ. There appears to be a steady increase in average number of shoots until the concentration of TDZ reaches to 0.5 mg/l and then, a slow decrease in mean number of shoots at higher TDZ concentrations (Figure 7).

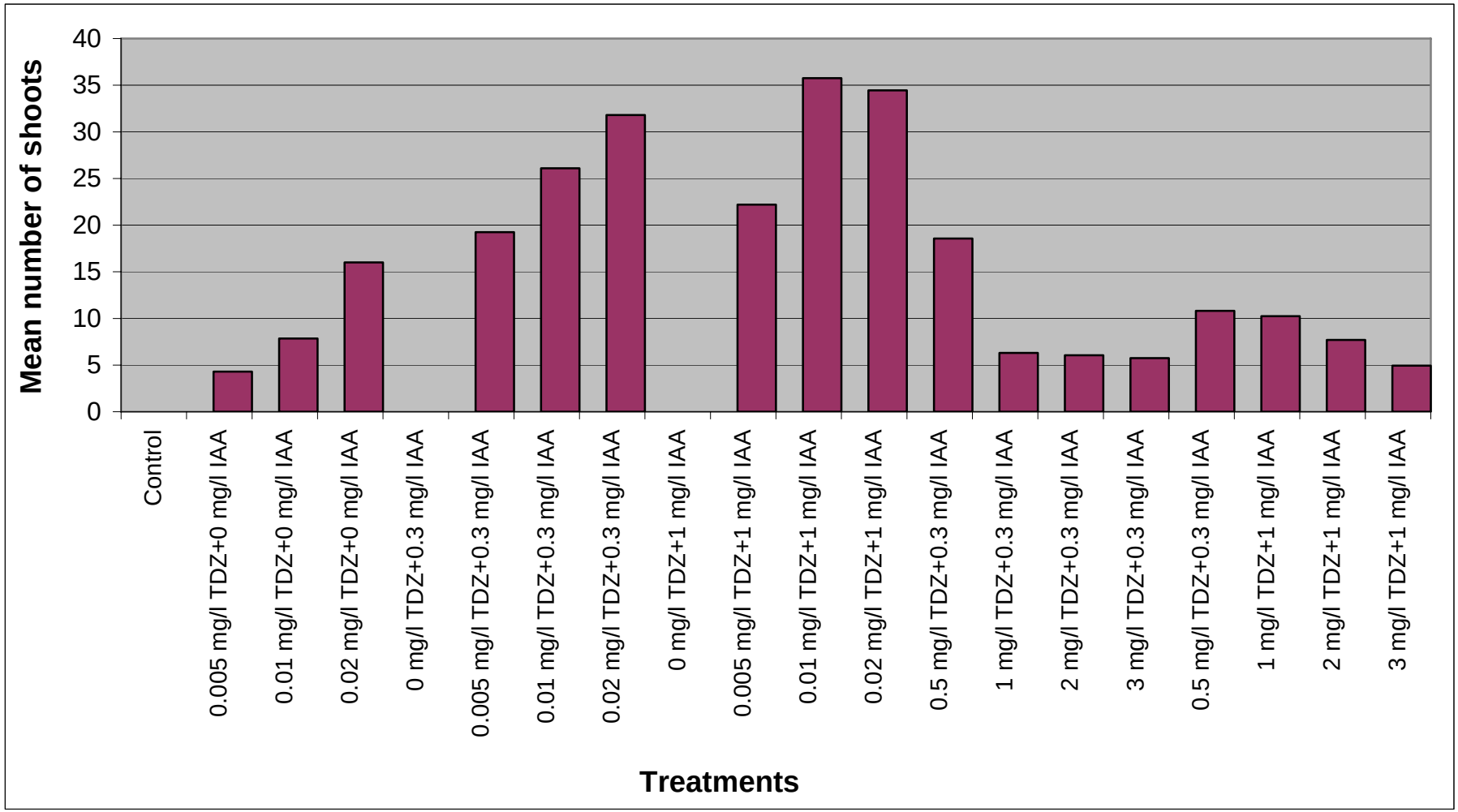
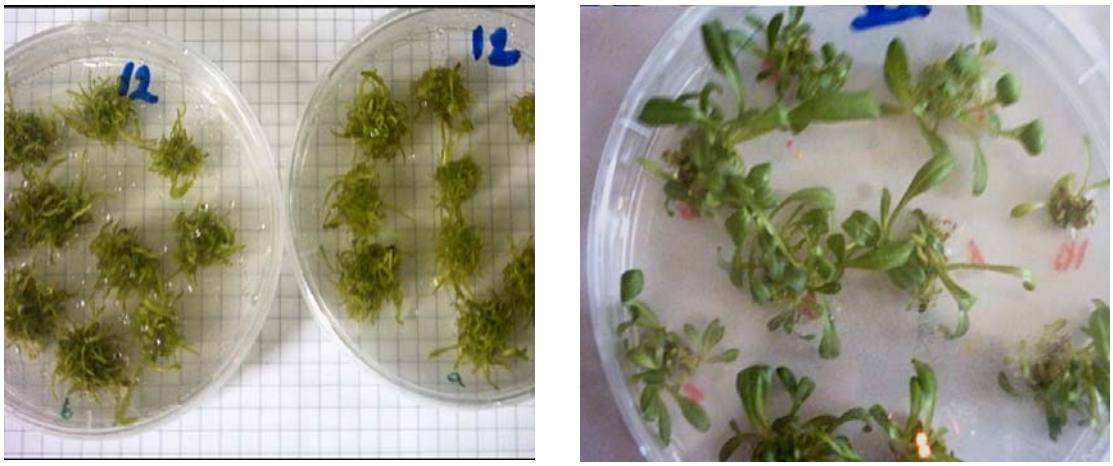



**Figure 7.** Mean number of shoots per explant on medium containing different concentrations of TDZ+IAA after 4 weeks culture.



**(a)** **(b)**

**Figure 8.** Comparison of leaf morphology in medium containing a) 0.02 mg/l TDZ+0.3 mg/l IAA, and b) 0.5 mg/l KIN+ 0.3 mg/l IAA.

## 3.2. Root Formation

Root formation was readily achieved from shoots, obtained from the previous experiments (Tables 5 and 6) using medium containing 0.3 mg/l IAA in combination with 0.5 mg/l KIN or 0.5 mg/l BAP or medium containing 1 mg/l IAA in combination with 0.005, 0.01 or 0.02 mg/l TDZ (Table 7). After removing the callus at the base of the shoots (3-4 cm in length, 4 weeks old), shoots placed into media containing different types of auxin (ie IAA, IBA, NAA or 2,4-D) at different concentrations, therefore, an alternative production of chicory root as a coffee substitute was optimized in our tissue culture protocols.

### 3.2.1. Effects of IAA and IBA on Root Formation

It is usual to induce root formation by increasing the auxin to cytokinin ratio of the culture medium. In terms of root formation, the media including IAA and IBA were used as the auxin rich media (Table 9).

Shooted explants from medium containing 0.3 mg/l IAA plus 0.5 mg/l KIN or 0.5 mg/l BAP was placed into the root induction media for four weeks. Mean number of root formation, shoot and root length per explant were observed. 0.5 mg/l IAA has the highest capacity for root formation per explant (4.24).

In the experiment, every 0.5 mg/l increase at IAA concentration almost halved the mean number of the roots and also lentgh of roots per explant (Figures 9 and 10). On the otherhand, among the medium containing various concentrations of IBA (treatments 5, 6 and 7) have no significant differences in terms of mean number of roots per explant, except treatment Treatment 1 (0.5 mg/l IAA) had almost 4 times higher root formation and mean root length than 0.5 mg/l IBA. In terms of callus formation during root induction, IAA had low callus formation. Data given in Table 9 was also analyzed using analysis of variance (ANOVA) method of Windows Excell program.

The diferences in mean number of roots per explant among treatments was found highly significant ($P= 9.03x10^{13}$), the lenght of root per explant was found very highly significant ($P= 1.55x\ 10^{23}$) and lastly, the mean length of shoots per explant was also found significant ($P=1.15x10^{12}$) at the 5% probability level.

Mean shoot length given in Table 9, also indicates the further development of the shoots during root induction. 0.5 mg/l IAA (Treatment 1) was a better source of seedlings for hardening off than other treatments (Figure 11). The explants on medium containing 2 mg/l IBA were weakest for root formation. In addition to 2 mg/l IBA, root formation using shoots on medium containing 1 mg/l IAA in combination with 0.005, 0.01 or 0.02 mg/l TDZ (Table 7) failed. Instead, only callus developed around the cut edges of the explants on medium contaning TDZ.

**Table 9.** Root formation when shoots, developed on medium containing IAA in combination with KIN or BA after a four week, were cultured on a rooting medium containing different concentrations of IAA or IBA alone. Mean values within columns followed by the same letter are not significantly different, $P<0.05$ (n=20 per treatment, experiment was repeated twice).

| Treatments | Mean Number of Roots per Explant | Percentage of Explants Forming Roots | Mean Length of Roots Explant (cm) | Mean Length of Shoots Explant (cm) | Callus Response |
|---|---|---|---|---|---|
| 1. 0.5 mg/l IAA | 4.24 ± 0.34 a | 100% | 6.64 ± 0.51 a | 6.31 ± 0.32 a | + |
| 2. 1.0 mg/l IAA | 2.67 ± 0.42 b | 85% | 3.43 ± 0.46 b | 4.33 ± 0.38 b | + |
| 3. 1.5 mg/l IAA | 1.15 ± 0.21 c | 65% | 1.93 ± 0.37 c | 4.30 ± 0.34 b | + |
| 4. 2.0 mg/l IAA | 0.68 ± 0.19 dc | 68% | 1.32 ± 0.29 c | 3.36 ± 0.40 cd | + |
| 5. 0.5 mg/l IBA | 0.92 ± 0.30 c | 56% | 1.54 ± 0.35 c | 3.62 ± 0.36 c | ++ |
| 6. 1.0 mg/l IBA | 1.50 ± 0.43 c | 68% | 1.73 ± 0.33 c | 4.16 ± 0.34 bc | ++ |
| 7. 1.5 mg/l IBA | 1.00 ± 0.15 c | 48% | 1.19 ± 0.33 cd | 3.52 ± 0.28 c | ++ |
| 8. 2.0 mg/l IBA | 0.55 ± 0.11 d | 45% | 0.78 ± 0.22 d | 2.90 ± 0.16 d | ++ |

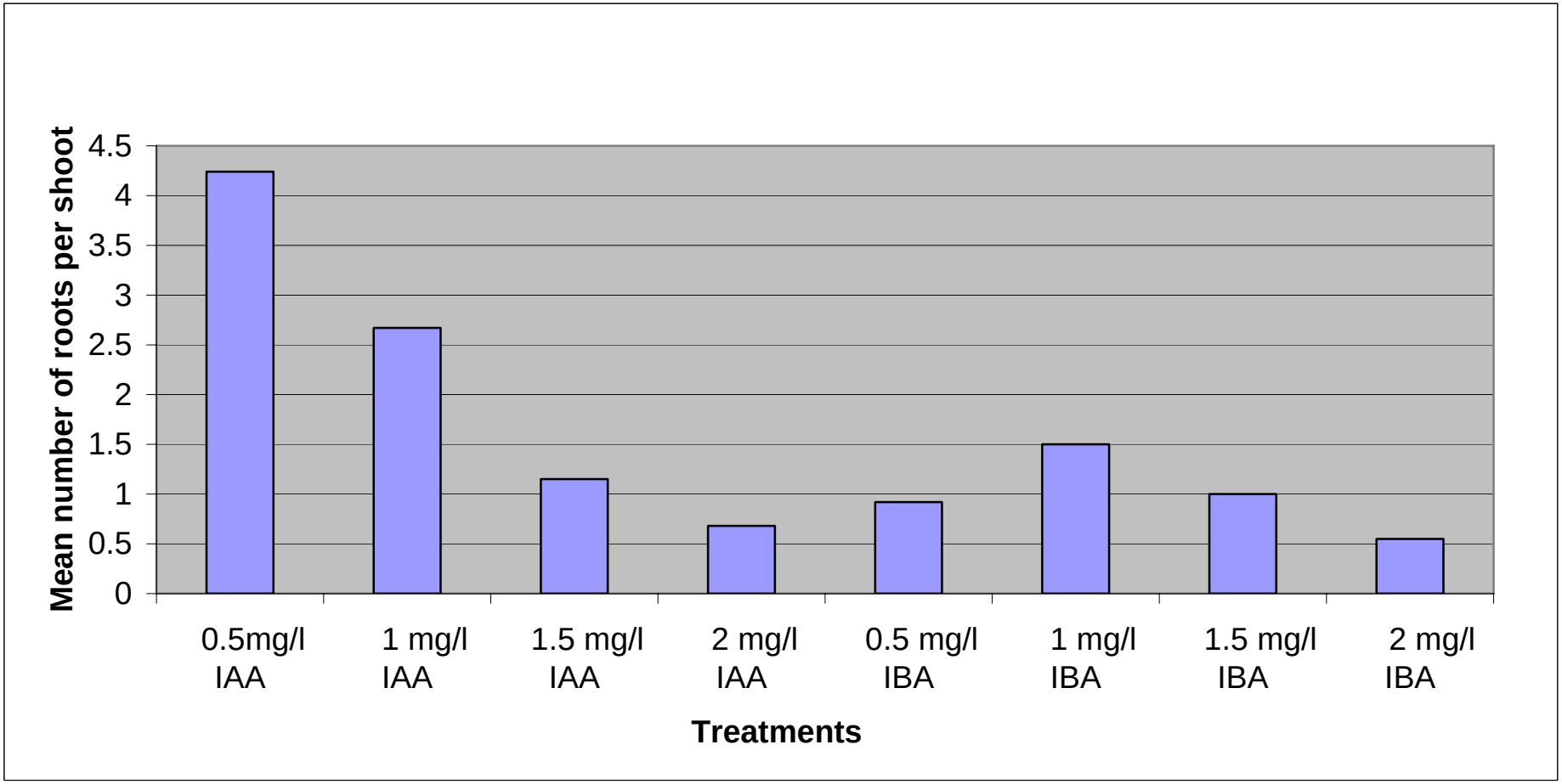



**Figure 9.** Mean number of roots per shoot on medium containing different concentrations of IAA or IBA after a four week culture.

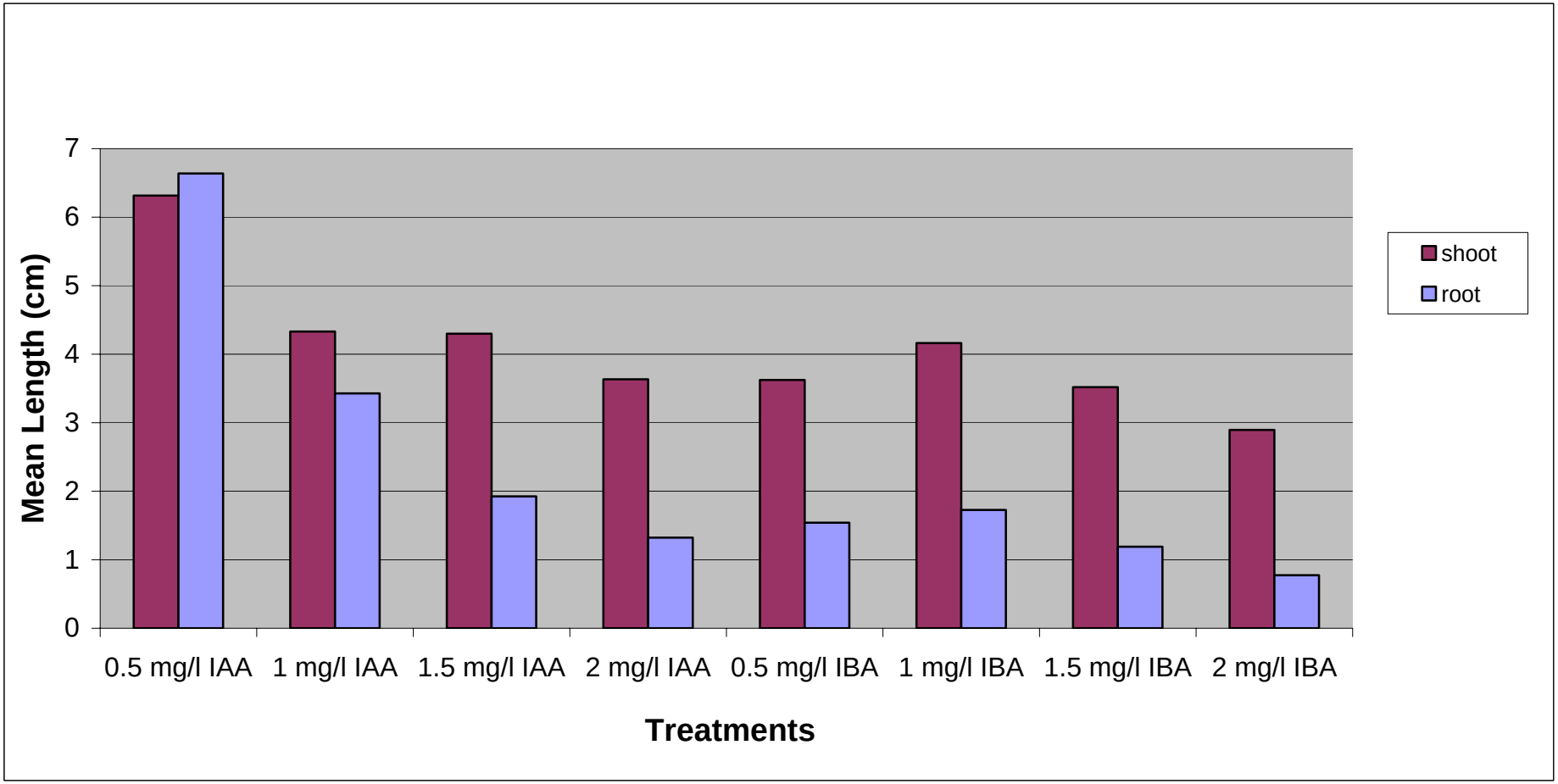



**Figure 10.** Mean shoot and root length per treatment. Measurements were done after four weeks' culture on root induction medium. For root length measurement, the longest root developed from the shoot explants was taken into account only.

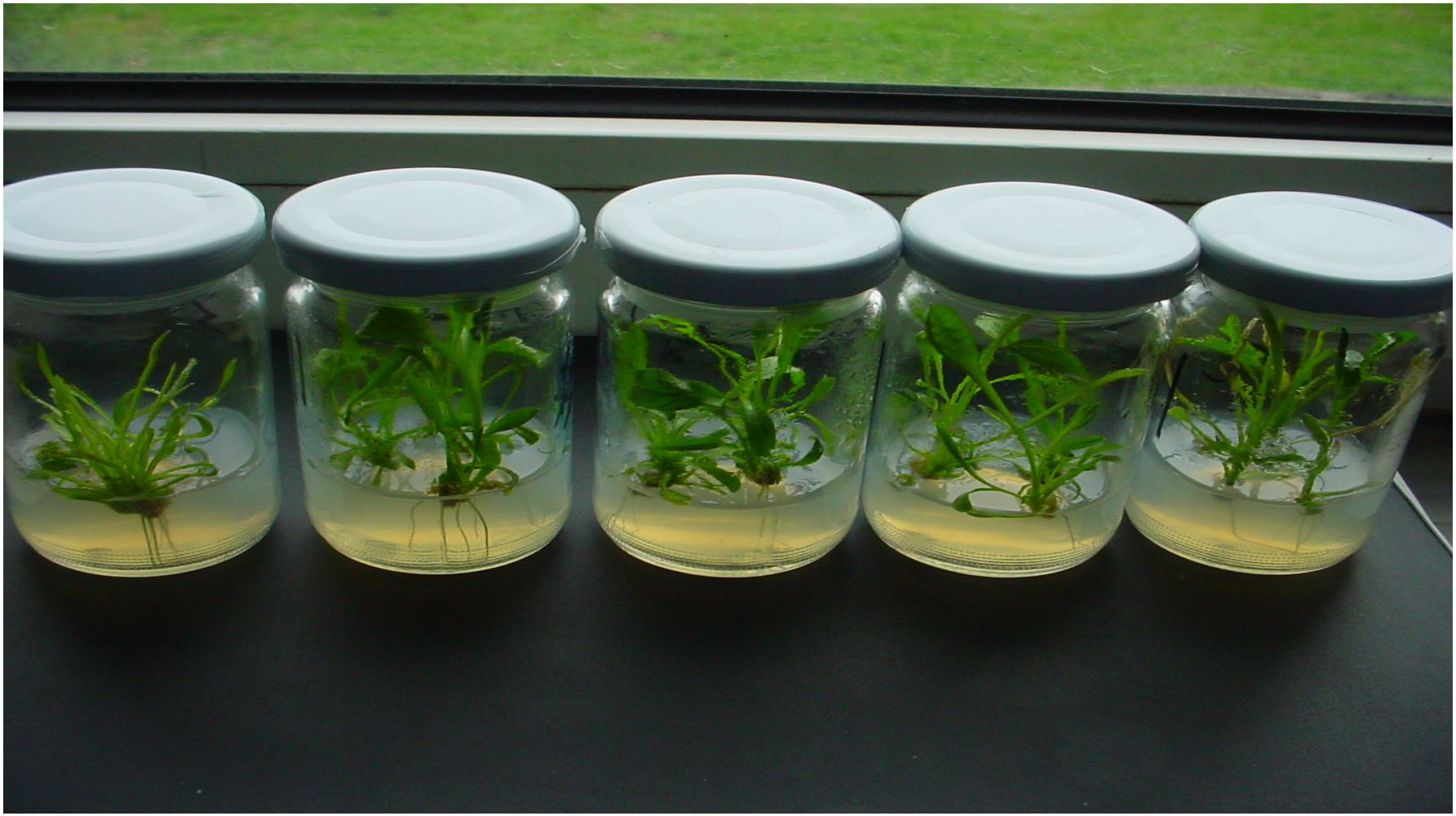


**Figure 11.** These explants were taken from Treatment 1 after four weeks culture.

### 3.2.2. Effects of 2,4-D and NAA on Root Formation

2,4-dichlorophenoxy acetic acid (2,4-D) and 2-1- naphthaleneacetic acetic acid (NAA) were also examined in addition to IAA and IBA for root formation. Root formation was found very poor in these two types of auxins. Rooting response was seen in 0.1 mg/l 2,4-D with 1.33 ± 0.42 root per explant and 0.1 mg/l NAA with 1.85 ± 0.26 root per explant (±SE, n=20). However, the increase in concentrations ranging from 0.5 mg/l to 2 mg/l for both types of auxins resulted in callus formation and reduced the growth and development of the seedlings in the jars and there was no root formation at highest concentrations of these hormones in any way.

There was not any significant difference in root number per explant between 0.1 mg/l NAA and 2,4-D at 5% probability level. The data showed that 0.5 mg/l IAA used in previous experiment had almost four times higher root number per explant than that of both 0.1 mg/l 2,4-D and NAA.

## 3.3. Hardening Off

After root formation, chicory seedlings were transferred to non-sterile conditions for acclimatisation and then to conditions of lower humidity levels progresively (Figure 12).

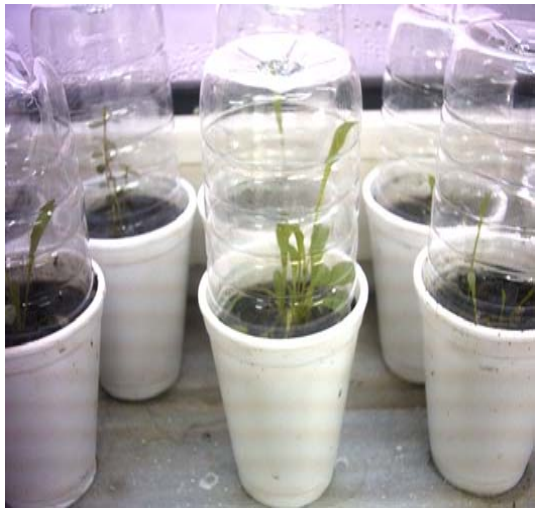
### 3.3.1. Conditions of Chicory Growth and Development

For hardening off stage, shoots that rooted on medium containin 0.5 mg/l IAA used (Table 9). Soon after autoclaving the commercial pot soil in a 3-liter capacity of autoclavable plastic container for 30 minutes, the soil was put into 150 ml capacity of foam pots (Figure 12).

After removing the agar residue around the roots, two chicory seedlings were placed into one pot then, the pots were drenched with tap water, and each pot including chicory seedlings covered with a transparent plastic cap (Figure 12-a), then transferred to the growth chamber at 23 °C and 55-60 % of humidity. The room humidity was provided by a humidifier (Figure 12-b).

The seedlings were watered until having the soil completely wet every 2 two days. Chicory regeneration was completed after 2 weeks in growth chamber room. Later, plastic caps on top of the pots were removed and pots were placed into the room conditions at which humidity and temperature was different (Figure 12-c).


**(a)**



**(b)**

**(c)**

**Figure 12** Some stages of hardening off, a) seedlings in the pots, b) in growth chamber and c) at the room conditions.

# 4. DISCUSSION

## 4.1. Preparation of Stock Material

*Cichorium intybus* L. is a widely used medicinal herb and coffee substitute. We, therefore, aimed at developing a reliable protocol for *in vitro* regeneration of this crop and thus examined the effects of several combinations and concentrations of plant growth regulator using leaf explants.

Our initial attempts to sterilize plant materials taken from field-grown plants (*ex vivo*) were unsuccesful due to significant contamination on the leaves. We then had to germinate seeds under aseptic conditions so that a sterile source of explant was obtanied continuously.

## 4.2. Effects of Plant Growth Regulators on Shoot Formation

Rehman et al. (2003) established the most recent study on *in vitro* regeneration of *Chicorium intybus* L. They found that mean number of shoots per leaf explant was 5.92 and percentage of the shoot formation was 84.4% on MS-semi solid basal medium containing 0.086 mg/l *(0.5 $\mu M$)* IAA in combination with 1.07 mg/l (*5 $\mu M$*) KIN in the presence of 1000 mg/l casein hydrolyzate (CH). In addition, they also examined shoot formation using 1.127mg/l (*5 $\mu M$*) BAP in combination with 0.086 mg/l IAA in the presence of 1000 mg/l CH and they obtained a mean value of 3.9 shoots per leaf explant and 61% of shoot formation. In terms of shoot formation, KIN seems more effective than BAP as we examined in our study. However, in terms of mean number of shoots seem quite different when compared their results with our data.

The mean shoot number per explant (5.92) obtained from the medium containing 0.086 mg/l IAA in combination with 1.07 mg/l KIN was approximately one-third of our result obtained from medium containing 0.5mg/l KIN plus 0.3 mg/ IAA. The differences in mean number of shoots could arise from basal medium used in studies were different. In terms of vitamin contents of MS and MSMO, myo-inositol and thiamine are considered essential in plant tissue culture media. Myo-inositol content of the MS and MSMO is 100 mg/l, however thiamine contents are different, that is MSMO contains 0.4 mg/l thiamine, while MS basal medium contains only 0.1 mg/l. Thiamine is also a B vitamin and is essential for carbohydrate metabolism and biosynthesis and thiamine has been also found to influence organogenesis in some plant cultures and myo-inositol takes a role as an intra-cellular messenger and enzyme activation (Rayns and Fowler, 1993,b). In addition to myo-inositol and thiamine, nicotinic acid and pyridoxine-HCl are present in only MS basal medium. According to Fowler and Rayns (1993,b), addition of nicotinic acid and pyridoxine-HCl is unnecesary due to the relatively high cost of vitamins, result in a considereble-wasted expense. It is not easy to decide which basal medium is better, there must be some particular cultures that may require the addition of other vitamis, whilst some do not require them. In our study, we only tested the *in vitro* regeneration of chicory using MSMO and showed a good particular response to shoot and root formation. Rehman et al. (2003) used MS- semi solid basal medium (gelling with 6 g/l agar) however in our experiment only MSMO was used and the medium was gelled with 8 g/l agar. Agar concentration in the medium is important during muliplication phase.

Gürel and Gülşen (1998) reported that leaf or leaf explants cultured at low agar levels (5 or 6g/l) were uptaken more water from the medium and therefore, exhibited much higher vitrification. Moreover, some of the ingredients in both MS and MSMO with different concentration of IAA plus KIN could cause significant differences in mean number shoots per explant. In our experiment, IAA concentration was approximately 4 times higher than what Rehman et al. (2003) used and KIN concentration was just about half of their KIN concentration. The succes of the shoot formation in our experiment was clear in terms of percentages of explants forming shoots (Table 4-5). 100% regeneration was found in our experiment, this value was almost 15% more than their results. It means that all explants in our study exhibited regeneration without vitrification or any death of the explant on medium containing 0.3 mg/l IAA in combination with 0.5 mg/l KIN. Concentrations of the hormone combinations most likely affected the mean value of shoot number and percentage of the shoot formations. In addition to the differences in basal media with various concentrations of plant growth regulators, they incubated the cultures at 27°C, whereas in our experiment, cultures were incubated at 23°C. Bais and Ravishankar (2001) reported that chicory required a moderate and uniform tempererature, with the optimum at 18 – 24°C for good plant growth in agricultural practises. Chicory leaf explants obtained *in vitro* performed rapid regeneration. In our experiment the shoot formation was observed via stereo microscope with 40 times magnification, therefore, total number of shoots per explant was considered using together with microshoots that were visible but uncountable by naked eyes. Counting the shoot numbers is relative to the observer, in our experiment explants that then differentiated into shoots either performed well-developed shoot formation or remained microshoots that would then become vitrified in some treatments, probably due to the lack of available nutrients and growth regulators in the medium.

This fact therefore should be overcome with subculturing (Gürel and Türker, 2001). All of treatments in our study were tested only once. This choice affected the number of explants for root formation later. Nevertheless, only one culture without subculture was available for later stages of our experiment when the short growth period of the chicory was considered. We also tested the effects of TDZ in combination with IAA for shoot formation in our experiment. Data showed that the low concentrations of TDZ increased the shoot formation (Figure 7). However, leaf morphology was not as good as expected. According to Heutteman and Preece (1993) the lower concentration of TDZ induces the shoot proliferation but may inhibit the shoot elongation. A comparison between the medium containing 0.02 mg/l TDZ plus 0.3 mg/l IAA and another medium containing 0.5 mg/l KIN plus 0.3 mg/l IAA was given in Figure 8. In terms of callus formation, Heutteman and Preece (1993) reported that higher concentration of TDZ in woody plants increased the formation of calluses. Although *Cichorium intybus* is a perennial herb or shrub, those of reported findings on some woody plants are consistent with our data. Further experiments are needed to overcome the leaf abnormality with addition of appropriate chemicals. Nevertheless, application of TDZ seems a good promoter of shoot formation (Chen et al., 2002; Ledbetter and Preece, 2004; Thomas and Philip, 2005) and somatic embriyogenesis in plant tissue culture (Singh et al., 2003). In our experiment, all leaf explants on medium containing NAA gave rise to callus formation much more than that of containing IAA. Park and Lim (1999) reported that as for growth and shoot initiation stimulant, the most potent auxin was IAA for the regeneration of chicory. According to our results the effects of NAA for callus initiation seemed preferable. Profumo et al. (1985) reported that NAA-type callus obtained from root explants was semi-compact, yellow-greenish, middle-sized cells. This finding was also similar to our results (Figure 6).

## 4.3.Effects of Plant Growth Regulators on Root Formation

In our experiment, 2,4-D used for root formation showed callus formation on the explants. At low concentration of 2,4-D (0.1 mg/l), produced 1.33 roots formation per explant. Any increase in concentration level of this hormone remarkably inhibited the root formation, besides; the growth of the shooted explants was reduced (Figure 13). On the otherhand, same pattern was found in the root induction media supplemented with NAA. Root formation was found at low concentaration of NAA (0.1 mg/L) as well as the root number per explant was found 1.85. Callus formation of the explants on medium containing NAA was more effective than 2,4-D (Figure 14). Profuma et al. (1985) reported that adventitious bud development was almost completely inhibited on 2,4-D medium and NAA at lower concentration promoted shoot formation. Furthermore, Rayns and Fowler (1993,c) reported that 2,4-D even at low concentrations, tends to result in callusing and has been linked to the genetic abnormalities. It was reported that activated charcoal enhance rooting by reducing illumination to the submerged parts of the culture (Rayns, 1993). Root quality of the chicory as a coffee substitute was also reported Bais and Ravishankar (2001), however, many disesases such as *Pseudomonas spp*. and *Erwinia spp* have been found major factors reducing root quality (Park and Lim,1999). Babaoğlu et al. (2001) reported that IAA had basic hormone structure and used very commonly. In our experiment, IAA as an auxin had the best response for root formation at 0.5 mg/l concentration level after 4 weeks initiation.

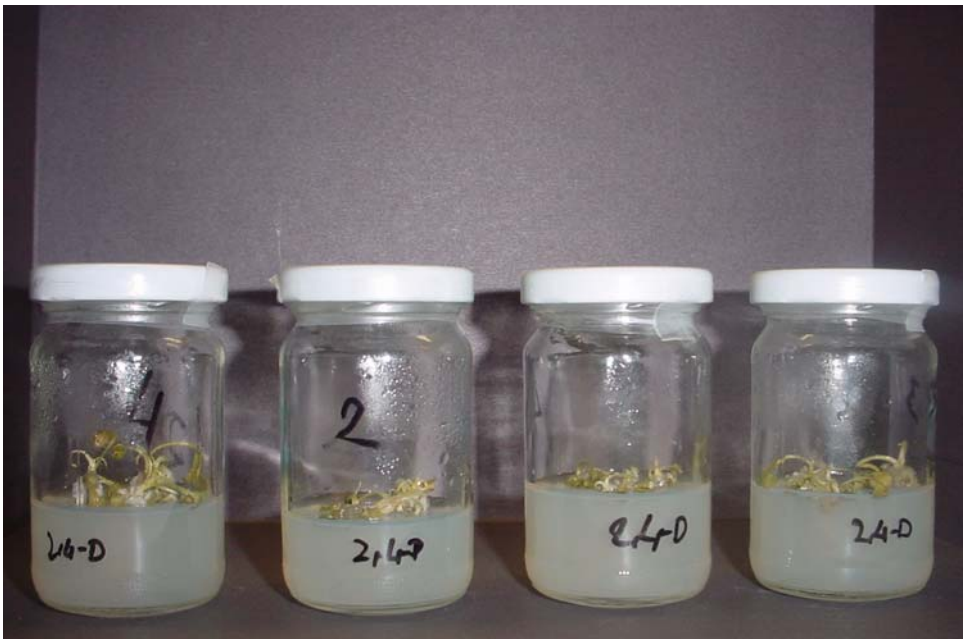



**Figure 13**. Reduction of shoot development on medium containing 2,4-D (0.1, 0.5, 1.0, 2.0 and 4 mg/l)


**Figure 14** Callus formation developed from the cut edge of the shooted explants on medium containing NAA at various in concentrations (jars left to right: 0.1, 0.5, 1.0, 2.0, and 4.0 mg/l)

## 4.4. Effects of Auxins on Shoot Development

During root formation, we also observed shoot development within the jars. Mean shoot length per explant was considered for each treatment (Table 8). We found that shoot development was inhibited in contrast to a gradual increase in auxin concentrations. Medium containing 0.5 mg/l IAA was found to have 6.31 cm shoot length per explant while the medium containing 2 mg/l IAA had 3.36 cm per explant. Same pattern was seen between 0.5 mg/l BAP having 3.62 cm shoot length per explant and 2 mg/l BAP having 2.90 cm shoot length per explant. The mean shoot lengths of both induction medium was significantly different; therefore, shoot development during root induction was repressed in the presence of excess amount of auxin in the media. Charriére et al. (1999) reported that auxin is generally considered necesarry for the acquisition of the embryogenic competence of the responsive cells. Once this competence has been established, excessive auxin concentrations are often inhibitory for further embriyonic development. It is likely that endegenous growth factors involved in the induction medium regulated in response to external culture conditions; therefore, our experiment may provide an understanding point for the mechanism of the auxin transport system.

## 4.5.Transfer to non-sterile Conditions

Shoots grown *in vitro* with or without roots are not adapted pysiologically to a survival in soil, therefore; they have to be gently weaned to accept such conditions. Rayns and Fowler (1993,a) indicated two major dangers for plantlets migth become dessicated and might be subjected to fungal rot. This fact can be explained in the following way; relative humidity in culture containers is usually close to 100%.

As a result, the plantlets do not neccesiate to regulate their transpiration rates. Consequently, their stomata remain open all the time and they recover the ability to close these stomata over a rather extended period. In this case, our plantlets were kept in growth chamber for two weeks.

In addition, transfer of such plantlets to soil in a relatively low humidity atmosphere resulted in dessication (Rayns, 1993). Therefore, we avoided this, providing high humidity (between 55-60%) to the room by a humidifier, besides; the plantlets were watered every two days. Since large plantlets with healty appearance were selected, we reduced the severe effects of fungal rot that formed due to the high humidity in the growth chamber.

# 5. CONCLUSIONS

Natural ecosystems regenerate themselves gradually during a period of time but almost agriculture and horticulture relies at some point on the establishment of fresh stock of plants. It must be remembered that most of our food comes from plants which are propagated by seed through sexual production.

In our study, chicory was propagated vegetatively *in vitro* after 8 weeks culture. At the end, we obtained much more fresh stocks of the crop than that of being yielded from agricultural practises. In general, vegetative propagation under lab conditions is expensive, thus only employed for high value crops where its advantages are particularly worthwhile.

Under these circumstances, chicory will be increasingly used in coffee blends, as a vegetable and in value-added healthcare products; many researchers believe that the next commercial secondary product process from plant culture *in vitro* will be based on an *Agrobacterium*-transformed system, thus; chicory can be chosen for future genetic manipulation due to its rapid regeneration *in vitro*.

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