IN VITRO INDUCTION OF GROWTH AND DEVELOPMENT OF COMMON JUNIPER (*Juniperus communis* L.) FROM SHOOT AND BUD EXPLANTS

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Approval of the Graduate School of natural and Applied Sciences

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ABSTRACT

IN VITRO INDUCTION OF GROWTH AND DEVELOPMENT OF COMMON JUNIPER (*Juniperus communis* L.) FROM BUD EXPLANTS

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The objective of the study was to investigate the optimum conditions for *in vitro* regeneration of common juniper (*Juniperus communis* L.) by using indirect organogenesis approach. Throughout the study; callus induction, organogenesis, improved organogenesis and root induction experiments were performed sequentially.

It was found that explant position, genotype, gender, treatments and sampling time had significant effects on callus induction rate in common juniper. The results of treatments indicated that IBA (indole-3-butyric acid) at concentration range 0.5-4.0 mg/l combined with MS medium supplemented with 0.1 mg/l BAP (benzylaminopurine), 3 % sucrose and 0.7% agar was the best one among the treatments to induce callus formation from common juniper explants collected as Spring buds. Also, a two-month culture was adequate period for the callus induction of common juniper regardless of position, before transferring the explants into organogenesis media.

After a two-month culture in callus induction media, explants were transferred to organogenesis treatments in order to investigate adventitious bud development from callus tissues. There were significant differences among genotypes, treatments and explant-sampling times in initiation of organ development in common juniper. Additionally, it was found that excluding the auxin components while maintaining 1.0-2.0 mg/l BAP concentration in culture media, as refreshing after a month, stimulated the formation and development of adventitious buds and shoots. Among the treatments tested, it was found that 1.0 mg/l BAP plus 0.5 mg/l 2,4-D was the optimum culture media with adventitious bud formation capacity of 37.5% was though ageing of callus significantly affected the frequency of adventitious bud formation.

Finally, rooting experiments were performed to investigate rooting efficiency of adventitious shoots. In the adventitious rooting experiments, no rooting was observed in any of the treatments used with common juniper explants.

Although whole plantlet development from callus tissues could not be achived as indirect organogenesis, the results of the study could aid to future studies dealing *in vitro* regeneration and production of secondary chemicals from common juniper.

Keywords: *Juniperus communis*; *in vitro* regeneration; indirect organogenesis; adventitious bud; adventitious shoot, callus induction

ÖZ

TOMURCUK EKSPLANTLARI KULLANARAK YAYGIN ARDIÇTA (*Juniperus communis* L.) BÜYÜME VE GELİŞMENİN *IN VITRO* KOŞULLARDA İNDÜKLENMESİ

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Bu çalışmanın amacı; dolaylı organ gelişimi metodunu kullanarak yaygın ardıcın (*Juniperus communis* L.) *in vitro* koşullarda rejenerasyonu için uygun koşulların araştırılmasıdır. Çalışma boyunca sırasıyla kallus indükleme, organ gelişimi, geliştirilmiş organ gelişimi ve köklendirme deneyleri yapılmıştır.

Eksplant pozisyonu, genotip, cinsiyet, besiyeri çeşitleri ve örnekleme tarihinin, kallus oluşumunda önemli etkiye sahip olduğu bulunmuştur. Deney sonucunda, uygulanan bütün besiyerleri arasında; 0.1 mg/l BAP (benzilaminopürin), % 3 sukroz ve % 0.7 agar içeren MS besiyerine ilave edilen 0.5-4.0 mg/l konsantrasyon aralığındaki IBA (indol-3-bütirik asit)'in Bahar döneminde toplanan yaygın ardıç tomurcuk örneklerinde kallus oluşumu için en uygun besiyeri olduğu bulunmuştur. Ayrıca, organ gelişimi besiyerine aktarmak için, eksplant pozisyonuna bakılmaksızın iki aylık kültür döneminin yaygın ardıçta kallus indüksiyonu için yeterli süre olduğu saptanmıştır.

İki aylık inkübasyon evresinden sonra, kallus dokularından adventif tomurcuk oluşumunu saptamak amacıyla, eksplantlar organ gelişimi için hazırlanan besiyerlerine transfer edilmiştir. Yaygın ardıç örneklerinde organ gelişimini tetiklemede, genotip, besiyeri çeşidi ve örnekleme tarihleri arasında önemli farklar olduğu bulunmuştur. Bunun yanı sıra, bir ay sonunda besiyerleri yenilenirken oksin bileşeninin besiyerinden çıkarılmasının 1.0-2.0 mg/l BAP konsantrasyonlarında adventif tomurcuk ve sürgün gelişimini arttırdığı saptanmıştır.Kallus yaşlanmasının adventif tomurcuk oluşumunu olumsuz etkilemesine rağmen; 1.0 mg/l BAP ve 0.5 mg/l 2,4-D içeren besiyerinin % 37.5 adventif tomurcuk oluşturma kapasitesi ile optimum kültür ortamı olduğu bulunmuştur.

Son olarak, adventif sürgünlerin köklenme başarısını saptamak amacıyla köklendirme deneyleri yapılmıştır. Bu deneyler sonucunda, yaygın ardıçta *in vitro* olarak kök elde edebilmek için uygulanan besiyerlerinin hiçbiri başarılı olmamıştır.

Dolaylı organ gelişimi uygulanarak kallus dokularından bütün bitki gelişimi sağlanamamış olmasına rağmen, bu çalışmanın sonuçları yaygın ardıçta *in vitro* rejenerasyon ve ikincil kimyasal üretimini konu alacak gelecekteki çalışmalara yol gösterecektir.

Anahtar Kelimeler: *Juniperus communis*; *in vitro* rejenerasyon; dolaylı organ gelişimi; adventif tomurcuk; adventif sürgün, callus indükleme

To my mommy, daddy and sister

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2,4-D	2,4-dichlorophenoxyacetic acid
2-IP	2-isopentenyladenine
ANOVA	Analysis of Variance
BAP	6-benzylaminopurine
HCl	Hydrogen chloride
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
KIN	Kinetin
MC	McCown Woody Plant Medium
MS	Murashige and Skoog basal salts
NAA	Naphthaleneacetic acid
NaOH	Sodium hydroxide
spp.	Species
subspp.	Subspecies

CHAPTER I

INTRODUCTION

Common juniper (*Juniperus communis* L.) is an evergreen, gymnosperm species. It is a perennial tree and usually found in dioecious form. This species is the most widely distributed tree throughout the world.

1.1 Characteristics of common juniper

1.1.1 Classification and genetic variation

Juniperus communis L., is belonging to the division *Coniferophyta* (conifers); class *Pinopsida* and order *Pinales*. It is also belonging to the family Cupressaceae (Cypress family) and the genus *Juniperus* (Davis, 1965).

Family Cupressaceae has been found in the fossil records since the Jurassic era. It includes 30 genera and 142 species. Phylogenetic tree in Figure 1.1 was derived from morphological characters rather than genetic data. Lineages in green represent genera formerly assigned to the Taxodiaceae; those in red represent out group comparisons (Farjon *et al.*, 2002).

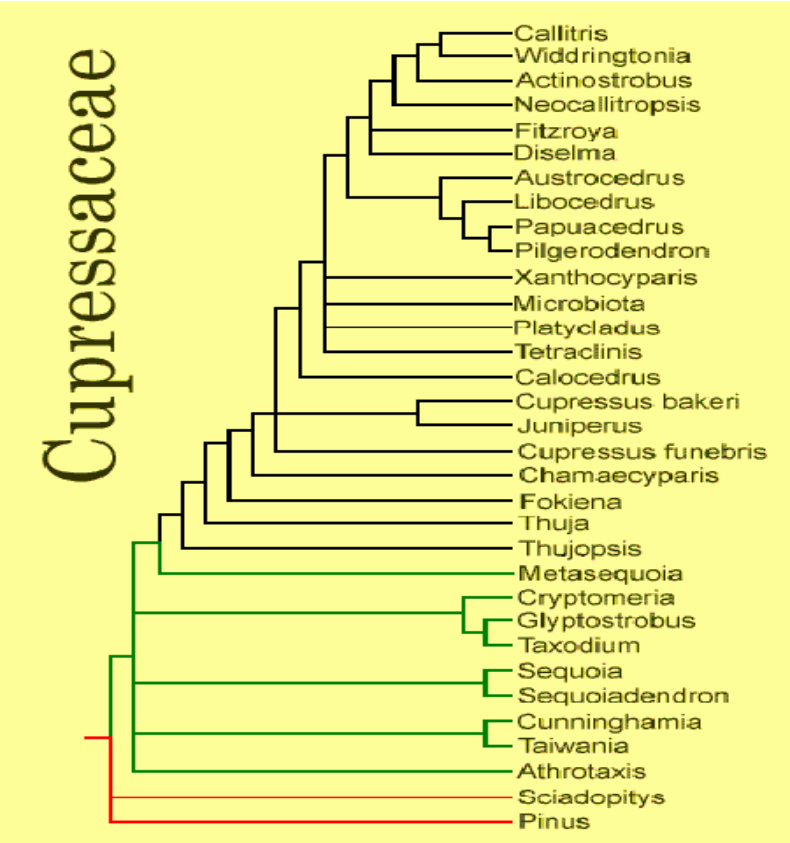


Figure 1.1 Phylogenetic tree for the Cupressaceae including all genera of the family (adopted from web page of Bonn University-available online at http://www.botanik.uni-bonn.de/conifers/cu/index.htm;

Last access date: 16.12.2004).

In studies of the systematics of genus *Juniperus*, terpenoids and Random Amplified Polymorphic DNAs (RAPDs) have been used to yield considerable results. Among these studies, many dealt specifically with *Juniperus communis* L. (Adams, 1998, 1999, 2000a, b, c, d, 2001; Adams *et al.*, 2002a; Adams *et al.*, 2002b; Adams *et al.*, 2003a).

The genus *Juniperus* includes approximately 68 species and 36 varieties (Adams, 1999, 2000a, b, c, d, 2001; Adams *et al.*, 2002a, b, 2003a, b). *J. communis* L. is the only species found in both hemispheres among other *Juniperus* species (Adams *et al.*, 2003a). According to the literature published, this genus is divided into three sections: *Caryocedrus* (one species, *J. drupacea* Labill.); *Juniperus* (= *Oxycedrus*, 14 species); and *Sabina* (the remaining approximately 55 species) (Adams, 2000a,b,c,d, 2001, 1999). *Juniperus communis* L. is belonging to the section *Juniperus* with respect to both terpenoids found in the leaf essential oils and RAPD DNA fingerprinting (Adams, 2000a).

With respect to RAPD markers, a *communis* complex consists of three groups: first group including var. *communis* and var. *saxitilis*; second group including var. *oblonga* and third group including var. *sibirica* (Adams, 2000a). Common juniper is a diploid species having chromosome number of 2n=22 (Flora of North America-available online at http://www.efloras.org/florataxon.aspx?flora_id=1&taxon_id=200005424).

1.1.2 Distinctive characteristics

Common juniper is an evergreen shrub or columnar tree. It can develop into a small tree of up to 7 m, but it is usually found as a multi-branched shrub less than 2 m in height. Its needle-like leaves with a white band on the upper face are the distinctive feature of this species. It is often confused with *J. conferta*, which has a green line down the middle of a narrow white stomatal line; and *J. oxycedrus*, which has two white stomatal bands on the upper face of the leaves. These needle-like leaves, which persist 3 years on the plant, are awl-shaped and have blunt needle tips. They are simple leaves and arranged in whorls of three. Younger leaves tend to be more needle–like whereas mature leaves are like scale-like (Davis, 1965; Kayacık, 1965; Tirmenstein, 1999).

(a) **(b)**

Figure 1.2 a) Branch and b) ripen fruits of common juniper (adopted from a) Henriette's herbal homepage-available at http://www.ibiblio.org/herbmed/pictures/p07/pages/juniperus-communis.htm, b) Josette Argaud web page-available at http://perso.wanadoo.fr/argaud/botanique/juniperus_communis.html,

Last access date: 16.12.2004).

The bark of common juniper thin, shredy or scaly, often exfoliating into thin strips. It is reddish brown in color and typically hidden by foliage. Its twigs tend to be yellowish or green when young but turn brown and harden with age (Figure 1.2a). Fruits are pea sized, globose, berrylike cones, which are red at first and turns into purplish-blue color while it ripens (Figure 1.2b). They are surrounded by an aromatic pulp. They contain 1-3 seeds in cones, which are found as sessile or on short stalks. The fruit matures in the autumn after a two-year period on the plant (Tirmenstein, 1999; Kayacık, 1965).

Figure 1.3 Schematic view of the parts of common juniper: A) male B) female cones (adopted from Kurt Stüber online library-available at http://caliban.mpiz-koeln.mpg.de/~stueber/thome/band1/tafel_023.html Last access date: 16.12.2004).

The wood of common juniper is fine grained, dense, durable, strong, and reddish with white sapwood. Since the juniper wood is a very slow growing tree, it has a dense structure without large resin canals. Therefore, due to the low accessibility of fluids to the core of the wood, the durability is increased. Furthermore, when compared with the hardwoods, low hemicellulose content in the tracheid wall of the softwoods of junipers makes them more chemically resistant than hardwoods (Gross & Ezerietis, 2003).

1.1.3 Chemical constituents

Common juniper contains notable quantities of essential oil in the timber, needles, berries and cones. This feature is not common in many trees.

Shahmir *et al.* (2003) reported that as making up 99.7% of the total composition of oils, 41 compounds were detected in needle's oil and 28 compounds were detected in berry's oil by gas chromatography-mass spectrometry (GC-MS). It was found that monoterpenes are the main components in both needle and berry oils of *J. communis* subspp. *communis*. Among these monoterpenes, terpinene-4-ol was found in the needle oil, while germacrene-D and limonene were found in the berry oil. They also observed that volatile oils are deposited in elongated tubercles. According to another study, it was found that the major compounds in the essential oils, extracted from ripe and unripe berries and leaves of some juniper species, were α-pinene, β-pinene, δ-3-carene, sabinene, myrcene, β-phellandrene, limonene and D-germacrene (Angioni *et al.*, 2003).

Besides volatile oil, common juniper also contains resin, bitter principle (juniperin), antitumour agent (podophyllotoxin), flavonoids, tannins, invert sugar and organic acids.

1.1.4 Reproductive biology of common juniper

Common juniper is a dioecious tree. Cones are ovoid to ellipsoid in shape and contain 1 to 3 seeds. Male strobili are sessile or stalked, and female strobili are made up of green, ovate or acuminate scales (Figure 1.4). Female cones enlarge while male strobili are shed. These strobili fuse to produce a berry-like cone. Female individuals bear axillary initial cones every spring. Although cone development generally occurs from April through June, its dates vary somewhat according to geographic location. These cones usually ripen from August through October of the second year and remain on the plant for at least 2 years. Seeds mature during the third year (Tirmenstein, 1999; Kayacık, 1965).

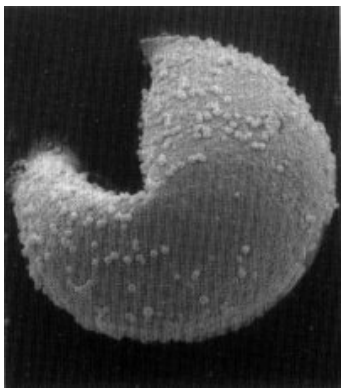


Figure 1.4 SEM photo of *Juniperus communis* pollen grain (adopted from the web page of University of London, Department of Geography-available at http:// www.geog.qmul.ac.uk/ popweb/junip/pollen.htm,
Last access date: 16.12.2004).

Development process from initial cone to ripe fruit occurs in three phases: 1) from spring of the first year to spring of the second year, pollination and delayed fertilization of the cone; 2) from spring to autumn of the second year, fruit growing phase (from a globular small green cone to a berry like fruit with green color and final size) during which the seeds begin to develop; 3) from the second autumn to the third autumn, fruit ripening and seed maturation. Color of fruit changes to blue gray in September. Since the cones are produced annually, it is possible to find both initial cones and fruits of two different cohorts simultaneously in the same plant (Garcia, 1998).

Seed dispersal is maintained by gravity, water, birds, or mammals. Birds are the most important dispersal agents for common juniper (Diotte & Bergeron, 1989). Digestive processes occurred in birds apparently do not harm most juniper seeds and may actually enhance germination (Emerson, 1932).

Due to the problems in germination, seedling establishment of common juniper is difficult. Ideal germination conditions are moist, compact soil with sufficient oxygen diffusion (Diotte & Bergeron, 1989). Depending on the seed source and specific treatment, germination has been reported to range from 7 to 75% (Tirmenstein, 1999).

1.1.5 Habitats and life forms

With respect to geographical location, phenotypic appearance and height of common juniper show some differences. Its height can reach 15 m in some locations while it is usually found in bush form in many places (Kayacık, 1965).

Common juniper grows on broad range of sites like dry, open, rocky, wooded hillsides, sand terraces, maritime escarpments, and on exposed slopes and plateaus throughout its range. Due to the intolerance of shade, it is usually found

in open environments (Diotte & Bergeron, 1989). Thus, this species can survive on different type of soils including acidic and calcareous sands, loams, or marls (Barkman, 1985). It is tolerant of ultramafic soils (Millar & Marshall, 1991). Common juniper is known as a colonizing plant especially on harsh, stressed environments in which competition lacks.

Common juniper is an indicator in a number of forest and shrubland habitat types and community types. It grows as a dominant with ponderosa pine (*Pinus ponderosa*), Douglas-fir (*Pseudotsuga menziesii*), lodgepole pine (*Pinus contorta*), limber pine (*P. flexilis*), white fir (*Abies concolor*), Engelmann spruce (*Picea engelmannii*), white spruce (*P. glauca*), quaking aspen (*Populus tremuloides*), blue spruce (*Picea pungens*), whitebark pine (*Pinus albicaulis*), subalpine fir (*A. lasiocarpa*), or Rocky Mountain bristlecone pine (*P. aristata*) (Tirmenstein, 1999).

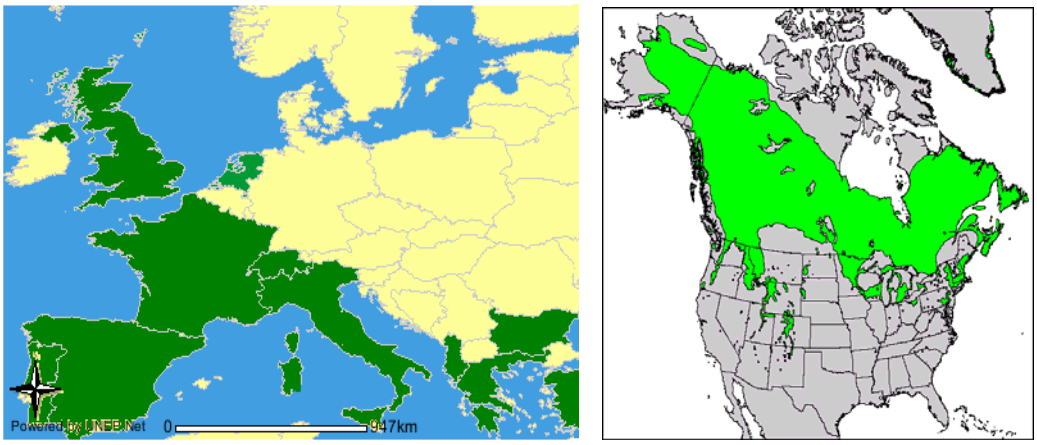
In Turkey, junipers are found in pure or mixed seed stands. In mixed stands, they are found with *Cedrus, Pinus, Quercus* and *Abies* species.

Since the foliage of this species is resinous, which is very flammable, common juniper is generally known as "susceptible" to fire. Thus, it is generally killed or seriously damaged by fire. If the fire occurs in patchy manner, then individual plants living on protected areas such as rocky cliffs may survive (Tirmenstein, 1999). In addition, lightly burned plant may also survive, if some portions are still alive; but it is a rare event (Stark & Steele, 1977). With increasing fire severity, the amount of damage increases; and therefore, regeneration potential of an individual plant decreases. After disturbance, common juniper does not sprout. It serves as a seed source for adjacent areas, if it still survives. Post-fire regeneration is proportional to the proximity to existing populations of common junipers (Diotte & Bergeron, 1989).

1.2 Distribution of common juniper in the World and in Turkey

1.2.1 Distribution in the World

Common juniper is a gymnosperm, which has the widest distribution in the northern hemisphere among other juniper species. Although it is isolated in the mountain areas of Mediterranean Europe (Garcia *et al.*, 1999), this species occurs across North America, New Mexico, Alaska, Europe, northern and central Asia (Caucasus, Iran, Himalaya, Kamchatka, Sahalin) (Figure 1.5), and Japan (Kayacık, 1965; Garcia *et al.*, 1999; Tirmenstein, 1999).



(a) **(b)**

Figure 1.5 Distribution maps of *J. communis* L. **a)** in Europe (adopted from web page of UNEP World Conservation Monitoring Centre-available at http://www.unep-wcmc.org) **b)** in North America (adopted from U.S. Geological Survey, 1999, Digital representation of "Atlas of United States Trees" by Elbert L. Little, Jr. –available at http://climchange.cr.usgs.gov/data/atlas/little/, Last access date: 16.12.2004). Green color on the maps indicates the species distribution in both Europe and North America.

1.2.2 Distribution in Turkey

In Turkey, there are 1.100.492 hectares of pure juniper forests, which are distributed at high mountain areas of nearly all regions (Figure 1.6). Main species found in Turkey are *J. drupacea* Lab., *J. communis* L., *J. oblonga* Beib., *J. oxycedrus* L., *J. phoenicia* L., *J. foetidissima* Willd., *J. excelsa* Beib. *and J. sabina* L. (Davis, 1965).

Figure 1.6 Distribution map of *J. communis* L. in Turkey (adopted from the web page of General Directorate of Forestry: http://www.ogm.gov.tr/agaclarimiz/agac9.htm,

Last access date: 16.12.2004).

1.3 Possible uses and economic value

1.3.1 Ecological value

In certain insular areas, *Juniperus communis* L. populations have serious problems in respect of regression (Ward, 1973; Clifton *et al.*, 1997). According to Garcia *et al.* (1999), as an indication of their regeneration process and their conservation status, age structure of the populations is important. Both remnant population dynamics and low resilience against disturbances decline the distribution area of *J. communis* L. in the high mountains of southern Spain after burning and clearing. In addition, due to Mediterranean summer drought, man-made attempts for the restoration of common juniper populations have failed.

According to Red Data Book of Turkish Plants (Ekim *et al.*, 2000), subspp. *communis* is considered as vulnerable species among the rare plants (not endemic) in Turkey. Besides this data, the community of common juniper is still under risk due to its low regeneration capacity, despite the species itself is not evaluated under the threatened/endangered category.

In Turkey, there are 12 gene conservation forests of *Juniperus* species, which covers an area of 2417,5 ha, among 163 gene conservation forests covering an area of 23408 ha for 24 different forest tree species. These forests are selected for protecting genetic diversity of forest tree species in their natural habitats and managed by special plans (Forest Tree Seeds and Tree Breeding Research Directorate, 2004).

Besides gene conservation forests, some individuals of *Juniperus* species are protected as natural monument. There are 7 juniper individuals among 58 natural monuments in Turkey (Table 1.1). They are also protected in some national parks in Turkey.

Table 1.1 Juniper trees as natural monument in Turkey (adopted from web page of Ministry of Culture, http://goturkey.kultur.gov.tr/turizm_en.asp?BELGENO=10143, Last access date: 16.12.2004).

Name	Place	Age (year)	Height (m)	Diameter (m)	Circum. (m)	Foundation date
Black juniper	Ankara	750	20	2.8	9	23.10.2000
Shah juniper	Antalya	800	24	2.35	7.38	21.02.1995
Lion juniper	Antalya	1700	25	3.4	12.53	21.04.1995
Kıranı Saint juniper	Gümüşhane	700	4.8	1.32	4.15	26.06.1995
Sögüt Plateau Great juniper	Isparta-Sütçüler	30	27	2.5	7.85	29.09.1994
Fossil juniper	Konya	500	-	-	4.5	27.09.1994
Mother juniper	Mersin-Tarsus	840	21	2.75	7.20	29.09.1994

With respect to their soil-retaining ability, their associated endemic flora and fauna, and their traditional use as summer grazing areas; these juniper shrublands have a high ecological value (Garcia *et al.*, 1999).

For short-term rehabilitation projects, common juniper has low value; on the other hand, it has moderate to high value for long-term rehabilitation projects. This species is highly valued as an ornamental. It provides good ground cover on different types of sites, even on stony or sandy sites. Thus, it is widely cultivated (Tirmenstein, 1999).

Common juniper also has potential as feed besides serving as nesting and covering for variety of animals. Such shrubs contain large amounts of tannins, which have most likely been evolved by these plants as a defense mechanism against being eaten by herbivores. Frutos *et al.* (2002) indicated that common juniper contains high amount of condensed tannin especially in reproductive tissues; flowers and fruits, which is potentially detrimental for herbivores.

From the ecological point of view, trees represent the main compartment of the ecosystem with respect to their biomass and nutrient contents. Montès *et al.* (2002) investigated the biomass and nutrient content of different parts of *Juniperus thurifera* L.. They showed that the mean biomass in the low-density juniper area was only 567 kg per tree but in most dense area, it can reach 1009 kg per tree. Furthermore, for the different parts, the nutrient concentrations (calcium, nitrogen, potassium and magnesium) indicates that Ca>>N>K>Mg in trunks/branches, branchlets and leaves, and K>N>Ca>Mg in female cones. In trunks or branches, significantly higher amounts of 71.2 kg ha $^{-1}$ for calcium and 43.5 kg ha^{-1} for nitrogen were detected. The nutrient content in leaves was significantly higher than that in branchlets and female cones for all elements. They also concluded that strong concentration of calcium in all tissues (except in reproductive organs) contributes in the neutralization of topsoil acidity through litter fall.

In Turkey, natural regeneration of juniper trees is widespread in forests. On the other hand, silvicultural precautions are not adequate for sustainability of juniper seed stands. Therefore, it results in a decline in juniper communities by narrowing its range and it would lead to disappearance of these juniper species found in Turkey.

1.3.2 Traditional use as a medicinal plant

Common juniper was first cultivated in 1560. Since then, common juniper has been used as a medicinal plant. Native Americans of the Great Basin used it as a blood tonic. Peoples living in different regions made tonics from the branches in order to treat a variety of ailments including colds, flu, arthritis, muscle aches, stomach and kidney problems. Its bark was used to treat respiratory problems (Turner, 1988).

Essential oil from common juniper has been traditionally used as an antiseptic for healing of the urinary tract problems, and as a diuretic in alcoholic beverages, especially, gin.

1.3.3 Biotechnological value

Different components of *Juniperus* species have possible biotechnological and industrial applications due to their chemical contents and wood structure. As mentioned in the previous sections, common juniper contains notable amount of tannins. Due to its traditional use by the indigenous peoples of the boreal forest in Canada for some symptoms of diabetes and its complications, McCune & Johns (2002) investigated the antioxidant activity of *J. communis* L. and some other medicinal plants. Tannin is a strong antioxidant itself and has the potential to cause liver damage. However, it has beneficial effects in digestion, the ability to detoxify some plant chemicals and action against tooth decay. Antioxidants overall proved to have considerable benefits in the prevention of the complications of diabetes.

Tunon *et al*. (1995) showed that the essential oil of common juniper possesses anti-inflammatory activity. Even at low concentrations (250 μg/ml); it has been shown that the oil extract sufficiently inhibits gram-negative and gram-positive

bacteria. Not all of the oils extracted from other species of *Juniperus* are beneficial. For instance, savin oil extracted from *Juniperus sabina* has abortifacient effects.

Today, common juniper has still been using as a flavoring in foods and alcoholic beverages such as gin. It has also been used as for flavoring liqueurs and bitters and as seasoning for pickling meats. It has been used as carminative and steam inhalant to treat bronchitis and used to control arthritis in the applications of herbal medicine (Shahmir *et al.*, 2003).

Another major use is in perfumery and cosmetics. All of the juniper extracts (oil or tar) have been especially obtained by steam distillation or alcohol extraction, and they are used as biological additives in cosmetic formulations, and *Juniperus oxycedrus* tar is used as fragrance component and a hair-conditioning agent. With respect to the acute studies using animals, the oil or tar showed little toxicity. In addition, *J. oxycedrus* tar and the oils from *J. communis* and *J. virginiana* were not skin irritants. *J. communis* oil was not phototoxic in animal tests, and *J. virginiana* oil was not a sensitizer. However, studies on albino rats demonstrated that *J. communis* extract could affect fertility and was abortifacient. (Johnson, 2001).

According to Stevensen (1998), in the application of aromatherapy in dermatological problems, essential oil from juniper berry is used as anti-inflammatory, analgesic, and used in the skin problems; and essential oil from juniper twig is used in the treatment of acne.

In the study of Goun *et al.* (2002), according to cytotoxicity assay results, nine plant extracts from Russia, including *Juniperus communis*, demonstrated 90% or higher activity in the inhibition of cancer L1210 (mouse leukemia) cells when extracted by methanol.

Since common juniper has a wood that can lie in water for a long periods of time without degradation, archeological findings have brought to light that the use of juniper wood as implements has dated back to the early 1800s (Larsen, 1990). Gross and Ezerietis (2003) investigated the use of a softwood, specifically *Juniperus communis* L., as an implant material for orthopedic applications; since similarity in micro-structural elements of the wood offer a possible candidate for implantation. In addition, with the additives of calcium, amorphous silica found in juniper wood is recognized as a bioactive material (Ducheyne & Qiu, 1999), which can facilitate osteoblast attachment. After 3 years of implantation, they found that the wood displayed good degradation resistance in the animal studies. The tracheids showed good results by establishing less middle lamellae, which binds the individual tracheids together. From this study, they concluded that *Juniperus communis* L. wood was a good material for implant in orthopedic applications, because, it contains an effective formulation of oil, that prevents infection. In addition, according to the toxicity studies, rats tolerated the oil well even at high doses than typically found in the wood implant. Furthermore, the use of boiling water for the sterilization of the wood lowered the elastic modulus, which provides better mechanical match of implant to bone. Rabbit studies also showed that the wood was well tolerated by the body and was surrounded by bone tissue. As a result, good bone attachment was observed (Gross and Ezerietis, 2003).

Another interesting application of *Juniperus* species is related with the removal of heavy metals from waste streams such as storm water runoff. Since its capacity to absorb heavy metals is relatively high when compared with that of other lignocellulosic fibers, *Juniperus monosperma* was used as plant material to absorb Cd^{+2} (Min *et al.*, 2004).

In their review, Merkle & Dean (2000) pointed out that by the aid of identification of naturally occurring mutants, as well as by engineering the lignin biosynthetic pathway with transgenes, radical alterations in the quantity

and quality of lignin in wood have been demonstrated to be possible in softwoods and hardwoods.

Since plants are also valuable sources for a variety of chemicals including drugs, flavors, pigments and agrochemicals; by the aid of plant cells, organ cultures and enzymes; a wide variety of chemical compounds including aromatics, steroids, alkaloids, coumarins and terpenoids can be produced by biotransformations (Giri *et al.*, 2001). According to the study of Muranaka *et al.* (1998), callus cultures of *Juniperus chinensis* produced low amounts (0.005% of dry weight) of podophyllotoxin and this production could be increased by 11-fold and 15-fold by the addition of phenylalanine, a biogenic precursor of podophyllotoxin and chito-oligosaccharides, an elicitor to calli, respectively.

1.4 Forest tree biotechnology

Since forest trees have undergone little domestication, biotechnology potentially has a greater impact on forest products and forestry than that on agronomic crops.

Forests are important for maintaining and preserving the ecosystem as well as to the world economy. In recent years, several molecular and biotechnological methods have been the focus of interest in forest tree research due to the decline in available harvestable forests. Major problem in traditional breeding of forest trees is their long generation time and their large size. Another handicap for the improvement by conventional breeding techniques is the high heterozygosity of forest trees that are especially propagated by seeds. For successful reforestation and forest management programs, it is necessary to consider large-scale clonal propagation of superior clones along with accelerated tree improvement programs.

1.4.1 History of tree tissue culture

In many of the early experiments, woody plants were the focus of interest since the eighteenth century in which callus production of wounded trees was observed by Duhamel du Monceau (Bonga & Aderkas, 1992). After that time, Schwann had also noted that in lower plants, plant cells have the capability to reconstitute the whole plant when any cell was separated from the plant. This observation had brought up the totipotency concept, which postulates that cells are autonomic, and capable of giving rise to a new plant (Bonga & Aderkas, 1992). These important observations enhanced attempts to regenerate plants from smaller tissue masses, and eventually, from single cells (Krikorian and Berquam, 1969).

For angiosperm tree species, the first regularly subcultured calli were those of *Salix caprea*, *Syringa vulgaris*, *Crataegus monogyna* and *Castanea vesca* with some of the calli producing a few shoots or roots. For gymnosperm species, the first callus maintained in continuous culture was that of *Sequoia sempervirens* (Bonga & Aderkas, 1992).

1.4.2 Major methods used in tree tissue culture

The primary goal of *in vitro* culture of forest trees has always been mass clonal propagation of the most desirable genotypes. Recently, it is also popular in obtaining target material for gene transfer to improve the quality of forest trees. Tree tissue culture dates back to 1934 when Gautheret studied first the callus induction from cambial tissues of several woody species (Vengadesan *et al.*, 2002). After this year, different regeneration systems have been studied and success has been achieved frequently. For *in vitro* propagation of forest trees, several techniques are available. Mostly used techniques are micropropagation, organogenesis, somatic embryogenesis, somaclonal variation and mutagenesis.

Superior offsprings are identified by the help of molecular markers and marker-assisted selection. In forest tree genome engineering, modification of lignin content and its composition, herbicide resistance, insect resistance, abiotic stress tolerance, flowering, restricting gene flow, altering tree form, quality and performance are the frontier areas (Tzfira *et al*., 1998).

Among these techniques, micropropagation is mainly used regeneration system in forest biotechnology. By this technique, it is possible to produce several millions of identical true-to-type individuals by saving time and space. Axillary shoot elongation, organogenesis and embryogenesis are the major methods of micropropagation.

Axillary shoot elongation is more common in commercial propagation of hardwood species than conifers, since it is the easiest method and it maintains genetic stability better than organogenesis. In this method, normally inactive axillary buds are released from apical dominance by adding of the hormones (primarily cytokinins) in the nutrient medium. The most frequent explant type is a short, single-node stem section for axillary shoot elongation (Bonga & Aderkas, 1992).

Organogenesis is used to the formation of shoots and roots. In this system, a cell or a group of cells differentiate to form organs, which is the reflection of the intrinsic genetic makeup of a taxon. In organogenesis, regeneration of shoots is focus of interest, since recovery of plants is the main objective. After obtaining shoots, root formation is induced by transferring to a different medium. Organogenesis occurs directly by the formation of shoots or roots from a pre-existing cell in the explant without undergoing an initial callus phase. On the other hand, if shoot or root induction and development are obtained through an initial phase of callus proliferation and growth by manipulating the application of exogenous phytohormone levels, then it is called as indirect organogenesis (Bonga & Aderkas, 1992).

Callus is a disorganized, proliferated mass of actively dividing cells and made up of a mass of loosely arranged thin walled parenchyma cells arising from the proliferating cells of parent tissue. It has potential to develop into whole plant by inducing shoots, roots or embryoids. There are several factors affecting the growth characteristics of callus: plant material, medium composition, environmental conditions during incubation period and time passing up to subculturing (Dodds & Roberts, 1985).

Callus formation occurs through three phases: induction, cell division and differentiation. In the first phase, metabolism is prepared for cell division. In the second phase, actively dividing cells of explant are reverted to meristematic or differentiated cells. Finally, third phase is the appearance of cellular differentiation and expression of certain metabolic pathways (Dodds & Roberts, 1985).

Somatic embryogenesis is the formation of an embryo from a cell other than a gamete or the product of gametic fusion. It has been reported in many woody trees. As opposed to other multicellular events such as organogenesis, somatic embryogenesis has a single cell origin. For the mass propagation of economically important plants, regeneration from cell suspension offers a suitable *in vitro* system. In 1960s, the first plantlets from forest tree tissue culture were obtained by adventitious shoots. However, in recent years, research efforts have been focused on *in vitro* propagation via somatic embryogenesis in respect of its advantages over other techniques. This technique has potentially high multiplication rate, and it is suitable for scale-up and delivery by bioreactor and for production of synthetic seeds. Furthermore, embryogenic cultures are also suitable for production of target tissue for gene transfer (Bonga & Aderkas, 1992). However, according to Merkle & Dean (2000), best of the embryogenic cultures lacks of commercial viability for two reasons: firstly, many of the most desirable clones have low frequency of regeneration; and secondly, most of the starting material for the cultures is derived from seeds or seedlings.

Scientists working in forest product companies have conducted much of the work related with conifer embryogenesis. In order to improve the higher plantlet production rate, researches with spruces and pines have focused on improving somatic embryo quality. According to the protocols already patented by industry researchers, to promote the highest production of mature *Pinus taeda* (loblolly pine) somatic embryos, treatments with abscisic acid, polyethylene glycol and maltose were applied (Li *et al*., 1998).

Another approach to tissue culture studies is the application of genomics. Cairney *et al.* (1999) tried to understand the developmental changes occurring *in vitro* at the gene expression level in order to improve regeneration from forest tree cultures in a more systematic manner.

1.5 Conventional and modern propagation methods for juniper species

1.5.1 Propagation by seed and cuttings

Juniper trees have critical problems in germination due to their dormant seeds. In order to overcome this dormancy, some studies have been conducted. According to The Colorado State Forest Service, summer sowing and "natural stratification" are beneficial for the production of rocky mountain juniper (*J. scopulorum* Sarg.). Stratified seeds are followed by natural soil regime over a seven-month period. By this way, 70% or better germination was achieved from Great Plains and Northern Colorado seed sources (Moench, 1995).

Common juniper is propagated mostly by the method of cuttings and by seeds. If the foliage is removed, then common juniper does not sprout well. On the other hand; when branches come in contact with the ground, adventitious root development can occur, and these roots aid in nutrient and water intake. As compared with the cuttings of common juniper from southern populations, those

from northern populations indicated better rooting capacity. It was also examined that cuttings from female individuals showed better rooting capacity than cuttings from male individuals (Houle & Babeux, 1994).

Berhe and Negash (1998) described procedures for the propagation of *J. procera* (African pencil cedar) through vegetative means and concluded that the most important factor controlling the rooting efficiency of cuttings was the age of stock plants. Negash (2002) collected the seeds from young individuals of *J. procera* and then seedlings were raised in a glasshouse. Then cuttings were collected and treated with different concentrations of indolebutyric acid (IBA) in order to determine rooting efficiencies of these cuttings. Results indicated that IBA concentration of 0.2% was suitable for rooting of cuttings derived from juvenile stock plants, as 0.4% IBA level was optimal for those derived from more mature stock plants.

Some of the other propagation studies by stem cuttings of different juniper species are listed in Table 1.2.

Table 1.2 Studies to optimize adventitious rooting from stem cuttings of different juniper species

Species	**Tested parameters**	**Reference**
Juniperus horizontalis Moench 'Wiltonii'	Medium moisture level	Rein *et al.*, 1991
Juniperus virginiana L.	nitrogen fertilization	Henry *et al.*, 1992a
Juniperus virginiana L.	Season, IBA application, genotype, crown position, type of cutting (straight vs. heel), cutting length, age of stock plant	Henry *et al.*, 1992b
Juniperus scopulorum	Effect of red or blue supplementary light	Bielenin, 2000

The results of these studies showed that, medium moisture level (Rein *et al.*, 1991) nitrogen (N) fertilization and levels of mineral nutrients boron (B) and potassium (K) (Henry *et al.*, 1992a); time of sample collection, IBA levels applied, genotype, type and length of cuttings and age of stock plant (Henry *et al.*, 1992b) influence the rooting efficiency of stem cuttings in different juniper species. On the other hand, supplementary red or blue light did not affect rooting of cuttings, but increased the root to shoot fresh weight ratio (Bielenin, 2000).

1.5.2 Propagation by *in vitro* techniques

To date, *in vitro* propagation of *Juniperus communis* L. has not been recorded. However, some tissue culture studies have been conducted in other species of genera *Juniperus*.

Gomez and Segura (1994) studied the morphogenetic capacity of mature *J. oxycedrus* L. leaves cultured *in vitro*; along with nutritive, hormonal and environmental factors inducing differentiation and development of adventitious shoots. According to their results, it was concluded that the highest bud differentiation rates were obtained by culturing the explants on a modified Schenk and Hilderbrandt (SH) solidified medium, containing 0.5 µM benzyladenine, for at least 21 days. They also stated that for maximum bud development and elongation, cytokinin-free medium containing 4% (wt/vol) sucrose and 0.05% (wt/vol) activated charcoal was appropriate. In order to induce root, regenerated shoots were cultured in the presence of 2.5 µM naphtaleneacetic acid and 4% (wt/vol) sucrose, and success was achieved by rooting percentages of up to 100%.

Gomez & Segura (1995a) developed a procedure for micropropagation of *J. oxycedrus* L. by using shoot apices and nodal segments from mature plants. For the shoot apices, best culture establishment was obtained by modified SH medium without growth regulators. However, for the shoot multiplication, regenerated shoots cultured in this medium were subcultured on SH medium including 0.5 µM benzyladenine. Unfortunately, success for root induction was not achieved.

In another study of Gomez and Segura (1995b) the effects of changes in the concentrations of macronutrients on BA-induced caulogenesis from leaves of mature *Juniperus oxycedrus* cultured on modified Murashige and Skoog or Schenk and Hilderbrandt media were investigated. For differentiation of

adventitious buds, the most favorable media formulations were those with nitrate:potassium, ammonium:potassium and nitrate:ammonium ratios near 1, around 0.1, and between 9-15, respectively. If disequilibrium of these ratios exists, then total ionic strength of the media limited the bud induction.

Embryonic explants of *Juniperus cedrus* Webb and Berth for plantlet regeneration was studied by Harry *et al.* (1995). By culturing whole excised embryos on Quoirin and LePoivre (QP) half-strength medium supplemented with 5 µM N-6-benzyladenine for 15 days, they induced an average of 6 buds per embryo. Then, explants were transferred to phytohormone free ½ QP medium for bud development. Shoots were elongated when explants were cultured on ½ QP with 0.05% activated charcoal and 2% sucrose. Finally, for the root induction, success was achieved when adventitious shoots were placed in pear-vermiculite-perlite (1:1:1) moistened with ¼ QP including 1% sucrose and 5 µM alpha-naphtaleneacetic acid under pH 5.0.

Negussie (1997) conducted a research on *in vitro* induction of multiple buds from the explants of *Juniperus excelsa*. In this study, excised cotyledon segments and embryo explants were cultured on both Eriksson (1965), and Murashige and Skoog basal media containing either 0.5 or 1 mg/l BAP with or without NAA. Results showed that Eriksson medium containing 0.02 mg/l NAA and 0.5 mg/l BAP was most suitable culture conditions for cotyledon segments on which 92 adventitious shoots per explant were obtained. For embryo explants, best response was achieved with MS medium including same concentration of NAA and 1 mg/l BAP. When adventitious shoots were pre-treated with IBA (1 mg/l) and NAA (0.5 mg/l) or activated charcoal for three weeks and then incubated in a covered seed tray filled with non-sterile compost for four months, better rooting response was achieved.

Cantos *et al.* (1998) studied the germination of intact seeds, seeds without testa and isolated embryos of *Juniperus oxycedrus* both *in vitro* and under

greenhouse conditions. They reported that intact seeds did not germinate in both conditions. However, although seeds without testa did not germinate under greenhouse conditions, those cultured on 1/3 strength of Murashige and Skoog medium supplemented with 3% sucrose and with or without 0.5 g/l GA_3 showed little response (12%) under *in vitro* conditions. On the same media composition, isolated embryos reached about 50% germination levels under *in vitro* conditions and acclimatization in greenhouse was very successful.

1.6 Justification of the study

Common juniper has great problems during its natural regeneration process. Major constraints for the natural regeneration of *J. communis* L., especially on Mediterranean mountains, are seed abortion, germination and seedling mortality (Garcia, 2001).

Since this species prefers harsh environments, where competition is lacking, in order to colonize, female plants decrease reproductive efforts under stressful conditions and thus, less viable seeds are produced (Marion & Houle, 1996). According to Pack (1921), up to 60% of common juniper seeds examined were defective. Therefore, low seed viability affects the germination rates of common juniper.

Since junipers are gymnosperms whose reproductive cones develop a fleshy parenchyma surrounding the seeds, they become attractive as a food material for the frugivorous animals. According to Garcia (1998), pest activity is clearly associated with a direct decrease in juniper reproductive capacity. In plants strongly attacked by some pests, the viability of seeds tended to be lower. When compared with the unattacked fruits, viable and unviable seeds were observed in lower proportions in fruits attacked by seed predators. Another reason for the low seed viability is the attack of pulp suckers, but this pattern is strongly

mediated by plant identity. As a consequence, since common juniper lives on harsh environments in which competition is lacking, plant reproductive trait variation may be the result of stressful environmental conditions such as drought and low nutrient availability, which make plants be more susceptible to herbivore attack. For the dispersion, except when plants are covered by snow, ripen cones are available from September through the winter and spring. However, many seeds in these ripe cones are either damaged by pre-dispersal seed predators or aborted during embryo development (Garcia, 2001).

According to Garcia *et al.* (2000); for *Juniperus* populations on Mediterranean mountains of south eastern Spain, high abortion rate is a typical feature. They concluded that this feature is related with climatic and genetic constraints during pollination and embryo development. Because embryos complete their development in a long period after cone ripening, a long dormancy is also common for the genus *Juniperus* (Johnsen, 1962; Chambers *et al.*, 1999). Since juniper seeds especially germinate during the two springs after dormancy period and no seeds germinate after this time, some scientists concluded that it is probably due to the loss of seed viability in the field after this two-year germination period (Holthuijzen & Sharik, 1984).

Another problem for the regeneration of common juniper is related with the characteristics of seeds. Since juniper seeds have a semi-permeable and thick seed coat with a dormant embryo, they require specific treatments before germination starts. Under natural conditions, germination rate of seed is generally only around 1% (Pack, 1921). Different types of treatments are used to induce germination of common juniper seeds including high temperatures, alternating temperatures, freezing and thawing, removal of seed coat, and application of hydrogen peroxide, dilute acids, carbon dioxide and light. However, it is found that all had little influence on the germination of juniper seeds (Tirmenstein, 1999). Mostly temperature treatments are used in order to enhance germination. Common juniper seed requires the application of warm

temperatures, followed by application of cold temperatures. This period lasts approximately 7 months (Diotte & Bergeron, 1989). As mentioned in the section 1.5.1, juniper seeds also require long stratification period to break the dormancy of seeds even under controlled conditions for germination.

A similar study was also conducted in Turkey. Alpacar (1988) reported a study about four different juniper species (*J. excelsa, J. foetidissima, J. oxycedrus* and *J. drupacea*), where the effect of different treatments on germination success of juniper seeds was investigated. It was found that majority of the seeds collected in the three years, during which the study was carried out, did not contain embryo. The most successful treatment (scarification followed by two months of warm and two months of cold stratification) produced about 50% germination success in only one species, but the results were inconsistent between years.

Due to the problems in natural regeneration of juniper tree, slow growing manner and absence in the rejuvenation studies, vegetative propagation methods for the regeneration of these species should be improved. For instance, with the help of micropropagation, rare genotypes could be cloned; thereby the chance of rare varieties being lost due to demographic stochasticity could be reduced. Furthermore, with a reliable and relatively fast way of regenerating individuals, the problems of overexploitation and overgrazing could be ameliorated by re-plantation of micropropagated individuals. By improving the tissue culture studies in juniper species, it is also possible to improve biotechnological applications mentioned in section 1.3.3. All these reasons justify the development of a procedure for *in vitro* propagation of juniper species.

This study is important by being the first *in vitro* testing of vegetative propagation ability of *Juniperus communis.* L. species.

1.7 Objective of the study

The objective of this study was to focus on *in vitro* propagation of *Juniperus communis* L. via indirect organogenesis. To determine *in vitro* conditions, the specific objectives were as follows:

- to induce callus formation by using the buds of common juniper
- to obtain adventitious buds from callus tissues
- to enhance shoot proliferation from the original bud explants and adventitious buds
- to induce adventitious root formation by culturing these newly emerged shoots.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Plant Material

The newly emerging shoots were collected from four individuals of common juniper trees located in the Middle East Technical University (METU) campus forest. Two of them were male and two of them were female trees (Figure 2.1). The individuals were 2-6 m in height. Two of individual trees were found in open environments while the other two were among black pine trees (*Pinus nigra*). Callus induction experiments were conducted with three sets by collecting the samples on November 2003 (sampling time 1), March 2004 (sampling time 2), and September 2004 (sampling time 3). While the first set of the experiment was composed of three genotypes, the other two sets included four genotypes. Organogenesis and organogenesis improvement experiments have not been completed with explants sampled at September 2004, yet. They were conducted with explants sampled at sampling times 1 & 2 (Table 2.1).

Figure 2.1 Male individual of common juniper

Table 2.1 Information on sampling times, number of genotypes and experiments

Experimental sets	Explant sampling times	Number of Genotypes	Callus induction experiments	Organogenesis experiments	Organogenesis improvement experiments
Set1	November 2003	3	√	√	√
Set2	March 2004	4	√	√	√
Set3	September 2004	4	√	X	X

2.1.2 Chemicals

All the chemicals used were plant tissue culture tested and purchased from Sigma-Aldrich Chemical Company (New York, USA) and Duchefa (Haarlem, The Netherlands). Tissue culture media used were Murashige and Skoog (MS) basal medium including all vitamins (Duchefa cat. no. M-0222), MS basal salts (Duchefa cat. no. M-0221) and McCown Woody Plant Medium (Sigma cat. no. M-6774). The growth regulators included 6-benzylaminopurine (BAP) (Sigma cat. no. B-3408), kinetin (Sigma cat. no. K-3378), 2-isopentenyladenine (2-IP) (Sigma cat. no. D-7660), indole-3-acetic acid (IAA) (Sigma cat. no. I-2886), indole-3-butyric acid (IBA) (Sigma cat. no. I-5386), 2,4-dichlorophenoxyacetic acid (2,4-D) (Sigma cat. no. D-7299), naphthaleneacetic acid (NAA) (Sigma cat. no. N-0640). Additionally, amino acid glycine (Sigma cat. no. G-7126), vitamins such as nicotinic acid (Sigma cat. no. N-0765) pyridoxine HCl (Sigma cat. no. P-8666), thiamine HCl (Sigma cat. no. T-3902), myo-inositol (Sigma cat. no. I-3011), as well as sucrose (Duchefa cat. no. S-0809), agar gel (Sigma cat. no. A-7002); and activated charcoal (Sigma cat. no. C-3790) were also used in the preparation of the tissue culture media.

2.1.3 Glassware

Glassware was purchased from Isolab-Interlab (Ankara, Turkey) and Sigma-Aldrich Chemical Company (New York, USA). Standard 10 cm diameter glass petri dishes (Isolab-Interlab) and baby jars (Sigma cat. no. V-0633 and V-8630) with autoclavable caps (Sigma cat. no. B-8648) were used.

2.1.4 Instrumentation

Laminar air flow (Bassaire, Southampton-England; Model 04HB) was used to carry out all aseptic procedures. Autoclave (Kermanlar, Istanbul-Turkey) was used to sterilize all nutrient media, distilled water and baby jars. Dry oven (Dedeoglu, Istanbul-Turkey) was used to sterilize petri dishes for 3 hours at 180^0C. Growth room was illuminated by cool-white fluorescent lambs (36watt x 2/shelf) for 16 h-daylight conditions. Temperature of the growth room was adjusted to 24-25^0C.

2.2 Methods

2.2.1 Explant preparation

2.2.1.1 Sterilization

Collected shoots were surface sterilized with 15% commercial bleach (Şok Market bleach) and 1-2 drops of detergent (Şok Market dish detergent) for 15 minutes. Then, samples were washed with sterile distilled water for four times to remove all chemicals. Each rinse was performed for 5 min. All explant manipulations were carried out in a sterile condition provided by a laminar flow hood environment.

2.2.1.2 Type of explant

As an explant, buds on newly emerging shoots were selected (Figure 2.2). Buds were excised from the shoots very carefully under aseptic conditions. After excision, these buds were transferred onto various nutrient media described in following section.

Figure 2.2 Bud explants of common juniper used in the callus induction experiments

2.2.2 Preparation of nutrient media and culture conditions

Various media preparation and culture conditions were set up for the callus and organogenesis (adventitious buds, shoots and root inductions) experiments. The details of these experiments were given in the following sections.

2.2.2.1 Callus Induction Experiments

For callus induction, 20 different treatments were applied. These were prepared by using MS based media including all vitamins (Murashige and Skoog, 1962). The MS media were supplemented with 0.1 mg/l BAP as cytokinin and four different auxin-type growth regulators (IAA, IBA, NAA and 2,4-D) at 5 different concentrations (0.5-1.0-2.0-4.0-10.0 mg/l) and 3% sucrose (Table 2.2). All media were solidified with 0.7% agar. The pH of the media was adjusted to 5.7 by the addition of NaOH or HCl in a drop wise manner. Then, all treatment-media were sterilized by autoclaving for 20 minutes at 121 ^{0}C under the pressure of 1.14 kg cm^{-2}. Since all the treatments were supplemented with MS

based media including vitamins, 3% sucrose and 0.1mg/l BAP, only auxin types and concentrations were given in Table 2.2.

Table 2.2 The growth regulator compositions of treatments for callus induction experiments

Treatments	Name of auxin type	Concentration of growth regulators (mg/l)
1	IAA	0.5
2	IAA	1.0
3	IAA	2.0
4	IAA	4.0
5	IAA	10.0
6	IBA	0.5
7	IBA	1.0
8	IBA	2.0
9	IBA	4.0
10	IBA	10.0
11	NAA	0.5
12	NAA	1.0
13	NAA	2.0
14	NAA	4.0
15	NAA	10.0
16	2,4-D	0.5
17	2,4-D	1.0
18	2,4-D	2.0
19	2,4-D	4.0
20	2,4-D	10.0

Excised buds were first transferred to these 20 different callus induction treatment-media under sterile conditions. For each of the media, two petri dishes were prepared. After medium preparation, explants were cultured in two different positions. Buds were placed in upward direction in one petri while they were in downward direction in the other petri. Five explants were placed in each petri dish.

After transferring the explants onto nutrient media, all cultured explants were incubated in growth room (see section 2.1.4) for two months. Nutrient media were refreshed monthly by subculturing of explants in freshly prepared treatment-media. Before each transfer to fresh media, data on the survival, callus initiation, callus size, and shoot formation were recorded (Table 2.3).

Table 2.3 Description of traits recorded in callus induction experiments

Traits	Description	Units
Survival (S)	Explant was either alive or dead	Count 1=alive 0=dead
Callus initiation (CI)	Explant started to develop callus	Count 1=callus initiation 0=no callus
Callus size (CaS)	Longest dimension of callus measured under microscope (1mm> callus induction)	centimeters (cm)
Shoot induction (SI)	Emerging of shoots from the original bud explants with or without callus at the bottom	Count 1=shoot induction 0=no shoot induction

2.2.2.2 Organogenesis Experiments

After two months of incubation in callus induction media, the explants were subjected to different sets of treatments to induce adventitious bud and shoot formations. For this purpose, 15 different treatment-media were applied (Table 2.4). These treatment-media were again MS based and including all vitamins (Murashige and Skoog, 1962). The treatment-media were supplemented with the following growth regulators: as a cytokinin type, BAP was used at 5 different concentrations (0.1-0.5-1.0-2.0-4.0 mg/l) and as an auxin-type 2,4-D was used at 3 different concentrations (0-0.5-1.0 mg/l). As in section 2.2.2.1, the media also contained 3% sucrose and solidified with 0.7% agar. The pH adjustment and sterilization of the media were also done as in section 2.2.2.1.

Calli and shoots from the callus induction experiments, if existed, were randomly transferred on to these 15 organogenesis-treatments. For each of the treatments, the nutrient media were prepared with three replications. All of the transfer procedures were done under sterile conditions. These calli were incubated in the growth room for a month. After this incubation period, the auxin in the medium compositions was removed as the media were refreshed; and the explants were incubated in these media again for another month. Conditions of the growth room were the same as explained in section 2.1.4.

Before each transfer, data about survival, amount of green (alive) callus tissue, meristemoid formation, number of adventitious buds and shoots from both original and adventitious buds were collected. Of these, amount of alive-callus-tissues were grouped into four categories as none (dead), low, medium and high alive-tissues. Meristemoid formation types were also categorized into four groups (none, low, medium and high). Adventitious shoot development types were grouped into six categories: none (closed bud), very little (closed but swollen bud), little (barely open bud with small shoot), good (an open bud with

a significant shoot), very good (larger shoot), perfect (shoots ~1 cm, with their own needles). The descriptions of all these traits were summarized in Table 2.5.

Table 2.4 The growth regulator compositions of treatments for organogenesis experiments

Treatments	Concentrations and names of growth regulators (mg/l)	
	BAP	**2,4-D**
1	0.1	0
2	0.5	0
3	1.0	0
4	2.0	0
5	4.0	0
6	0.1	0.5
7	0.5	0.5
8	1.0	0.5
9	2.0	0.5
10	4.0	0.5
11	0.1	1.0
12	0.5	1.0
13	1.0	1.0
14	2.0	1.0
15	4.0	1.0

Table 2.5 Description of traits recorded in organogenesis experiments

Traits	Description	Units
Survival (S)	Explant was either alive or dead	Count **1**=alive **0**=dead
Green callus (GC)	Amount of alive callus tissue	Class **0**=none (dead) **1**=low **2**=medium **3**=high
Meristemoid formation (MF)	Amount of meristemoid formation on calli	Class **0**=none **1**=low **2**=medium **3**=high
Adventitious shoot development (ASD)	Size of emerging of shoots from both original and adventitious buds	Class **0**=none (closed) **1**=very little (closed but swollen) **2**=little (barely open with small shoot) **3**=good (open with a significant shoot) **4**=very good (larger shoots) **5**=perfect (shoots~1 cm with own needles)

2.2.2.3 Organogenesis-Improvement Experiments

In order to see the effects of different cytokinin and auxin types, the spectrum of treatments applied in the first phase of organogenesis was extended further. Thirty-six different treatments were designed in the second phase of organogenesis. Media compositions were designed as in section 2.2.2.2. These media were supplemented with one of three types of cytokinin (BAP, Kinetin, or 2-IP) at 3 different concentrations (0.5-1-2 mg/l) and one of two types of auxin (2,4-D or IBA) at 2 different concentrations (0.5-1 mg/l). Media compositions were summarized in Table 2.6. Media were sterilized again as in section 2.2.2.1.

Table 2.6 The growth regulator compositions of treatments for improved organogenesis experiments

Treatments	Concentrations and names of growth regulators (mg/l)	
	BAP	**2,4-D**
1	0.5	0.5
2	1.0	0.5
3	2.0	0.5
4	0.5	1.0
5	1.0	1.0
6	2.0	1.0
	2-IP	**2,4-D**
7	0.5	0.5
8	1.0	0.5
9	2.0	0.5
10	0.5	1.0
11	1.0	1.0
12	2.0	1.0
	Kin	**2,4-D**
13	0.5	0.5
14	1.0	0.5
15	2.0	0.5
16	0.5	1.0
17	1.0	1.0
18	2.0	1.0
	BAP	**IBA**
19	0.5	0.5
20	1.0	0.5
21	2.0	0.5

(continued)

22	0.5	1.0
23	1.0	1.0
24	2.0	1.0
	2-IP	**IBA**
25	0.5	0.5
26	1.0	0.5
27	2.0	0.5
28	0.5	1.0
29	1.0	1.0
30	2.0	1.0
	Kin	**IBA**
31	0.5	0.5
32	1.0	0.5
33	2.0	0.5
34	0.5	1.0
35	1.0	1.0
36	2.0	1.0

After two-month incubation of explants in the first phase of organogenesis media, all the buds and shoots were transferred on to the rooting media while calli were randomly transferred on to these 36 treatments. With respect to the calli size, large calli were divided into smaller pieces when subculturing was practiced. For each treatment, the media were prepared with two replications. These calli were incubated in growth room for two months (see section 2.1.4 for the growth room conditions) and nutrient media were refreshed monthly. Before each transfer, data were recorded in respect to traits described as in Table 2.5.

2.2.2.4 Adventitious Rooting Experiments

After the incubation period in the first phase of organogenesis media, both original buds and emerging shoots including newly emerged adventitious shoots from the calli during experiment were transferred to the rooting medium. By this time, explants were 4-months old.

For root induction, several different medium compositions were tried sequentially. The medium compositions were as follows (in the order they were applied): 1) MS basal salts supplemented with four different concentrations of IBA (0-0.005-0.030-0.050 mg/l) and activated charcoal, 2) ½ McCown (MC) woody plant media supplemented with 0.25 mg/l IBA, 3) MC woody plant media supplemented with 0.05 mg/l IBA. All media were prepared by adding 100 mg/l myo-inositol, 2 mg/l glycine, 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine HCl, 0.1 mg/l thiamine HCl, 3% sucrose, and 0.7% agar. The pH of the media was adjusted to 5.7. Then all media and baby jars were sterilized by autoclaving for 20 minutes at 121 ^{0}C under the pressure of 1.14 kg cm^{-2}. In Table 2.7, the medium types and IBA concentrations were given in detail.

Table 2.7 The growth regulator compositions of treatments for adventitious rooting experiments

Treatments	Medium type	Concentrations and name of growth regulator (mg/l)
1	MS basal salts	No-0.005-0.03-0.05 IBA
2	½ MC woody plant medium	0.25 IBA
3	MC woody plant medium	0.05 IBA

Four-month old buds and shoots from the organogenesis experiment were transferred into baby jars and incubated in the first treatment in Table 2.7 for another two months. After that, they were subjected to the second treatment-medium in Table 2.7 for one month, followed by two months in the third treatment-medium in Table 2.7. Incubation conditions were the same as in section 2.1.4.

Again, before each transfer, data were recorded on survival of explants, adventitious shoot development as described in Table 2.5, and presence or absence of adventitious root initiation.

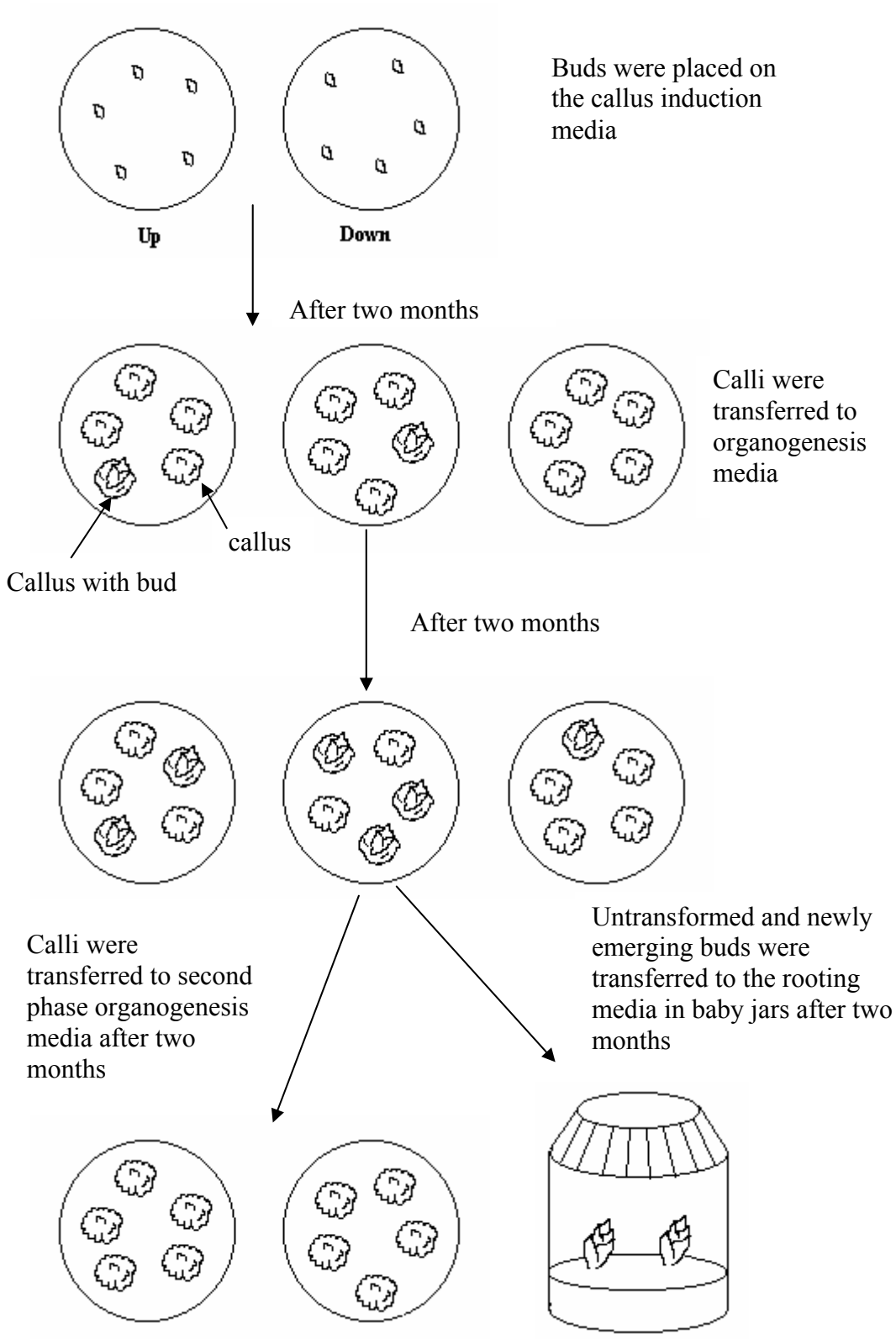


Figure 2.3 Schematic representation of the experiments carried out for induction of callus and organogenesis in common junipers

2.3 Statistical analyses

All of the statistical analyses were carried out by using Minitab software, version 13 (Minitab, Inc., 2000). Fully nested analysis of variance (ANOVA) with General Linear Model (GLM) was used to examine the effects of multiple factors (Replications, Genotypes and treatments) on response variables (callus, adventitious buds and shoots, roots etc). All factors in a fully nested ANOVA were assumed to be with random effects. The variance components due to genotypes and treatments were estimated by using the expected mean squares in Table 2.8.

The treatment means for callus induction, adventitious buds and shoots were calculated again using the mean procedures of Minitab. Standard errors of means were also estimated and given in appropriate tables in Appendix C. The treatment means were also given in graphical forms by using Microsoft Office Excel (2000) to explore the genotype and treatment effects on recorded callus and organogenesis traits.

Table 2.8 Forms of expected mean squares (MS) and appropriate *F* tests for the studied traits in Callus Induction (A), Organogenesis (B) and Organogenesis Improvement Experiments (C) (df=degrees of freedom)

Experiments	Source of Variation	df	Mean Squares	Appropriate *F* tests	Expected Mean Squares
A	Replications	2	MS_1	$F=MS_1/MS_2$	σ^2_e +8,9 $\sigma^2_{T(G)}$ +164,6 σ^2_G + 601,7 σ^2_R
	Genotypes	3	MS_2	$F=MS_2/MS_3$	σ^2_e +9.0 $\sigma^2_{T(G)}$ +169.0 σ^2_G
	Treatment (Genotypes)	76	MS_3	$F= MS_3/MS_4$	σ^2_e +8.5 $\sigma^2_{T(G)}$
	Error	1770	MS_4		σ^2_e
B	Replications	1	MS_1	$F=MS_1/MS_2$	σ^2_e +5,3 $\sigma^2_{T(G)}$ +73,3 σ^2_G + 244,3 σ^2_R
	Genotypes	3	MS_2	$F=MS_2/MS_3$	σ^2_e +5,1 $\sigma^2_{T(G)}$ +68,5 σ^2_G
	Treatment (Genotypes)	55	MS_3	$F=MS_3/MS_4$	σ^2_e +4,8 $\sigma^2_{T(G)}$
	Error	429	MS_4		σ^2_e
C	Replications	1	MS_1	$F=MS_1/MS_2$	σ^2_e +1,8 $\sigma^2_{T(G)}$ +53,2 σ^2_G + 167,8 σ^2_R
	Genotypes	3	MS_2	$F=MS_2/MS_3$	σ^2_e +1,7 $\sigma^2_{T(G)}$ +45,9 σ^2_G
	Treatment (Genotypes)	125	MS_3	$F=MS_3/MS_4$	σ^2_e +1,6 $\sigma^2_{T(G)}$
	Error	206	MS_4		σ^2_e

CHAPTER III

RESULTS AND DISCUSSION

This study was concentrated on the *in vitro* regeneration of common juniper via indirect organogenesis by which adventitious buds, shoots and roots can be obtained through callus tissues. In the study, buds from newly emerging shoots of mature individual trees were selected as a source of explants. Callus induction, organogenesis and improved organogenesis, and adventitious rooting experiments were carried out sequentially to obtain plantlets by vegetative means.

3.1 Callus Induction Experiments

The induction of callus growth and subsequent differentiation are maintained by the differential applications of growth regulators and the control of conditions in the culture medium. Cell division, cell growth and tissue differentiation are induced with the stimulus of endogenous growth substances, or by addition of exogenous growth regulators to the nutrient medium.

In the callus induction experiment, callus initiation capacity of the common juniper explants were determined by recording data on the callus initiation (CI) and callus size (CaS). Additionally, survival (S) and adventitious shoot induction (SI) rate of the explants were also considered (see Table 2.3 for the trait descriptions). This part of the study was conducted with three sets by collecting the samples at three different times (Table 2.1). First set of the experiment consisted of the samples from three individuals (genotypes) while the other two sets included that of four individuals.

With respect to survival, callus initiation, callus size and shoot induction traits, the output of the results of data set treated as *Fully Nested ANOVA* and analyzed with General Linear Model procedure of the Minitab Statistical Software. The results were provided in Table 3.1 via using adjusted mean squares. In addition, Pearson correlation values between traits were estimated for callus induction experiments and the results were given in Table 3.2. In the following sections, effects of position, genotypes, treatments and sampling times on the induction of callus from bud explants of common juniper will be explored with the experimental data.

Table 3.1 ANOVA table for the traits considered in the callus induction experiments (MS: adjusted mean squares; VC: variance component; df: degrees of freedom)

Traits	Replication df=2	Genotype df=3		Treatment (Genotypes) df=76		Error df=1770	
	MS	MS	VC%	MS	VC%	MS	VC%
Survival	5.158	1.650	2.38**	0.253	4.05**	0.127	93.57
Callus initiation	7.849	2.422	2.97**	0.395	6.93**	0.142	90.10
Callus size	1814.430	1269.090	12.86**	62.950	9.25**	16.810	77.89
Shoot induction	6.704	2.428	6.12**	0.233	8.52**	0.071	85.36

*significant at $p< 0.05$; **significant at $p< 0.01$

Table 3.2 Pearson correlations between traits recorded in callus induction experiments (see Table 2.3 for the traits descriptions)

	Position	**M1 S**	**M1 CI**	**M1 CaS**	**M1 SI**	**M2 S**	**M2 CI**	**M2 CaS**
M1 S	0.093**							
M1 CI	0.441**	0.206**						
M1 CaS	0.206**	0.170**	0.726**					
M1 SI	-0.054*	-0.028ns	-0.054*	-0.013ns				
M2 S	0.131**	0.382**	0.326**	0.278**	-0.024ns			
M2 CI	0.199**	0.191**	0.599**	0.436**	-0.043ns	0.425**		
M2 CaS	0.029ns	0.176**	0.530**	0.801**	-0.000ns	0.356**	0.580**	
M2 SI	-0.137**	0.087**	-0.134**	0.100**	0.276**	0.117**	-0.040 ns	-0.022 ns

*significant at $p< 0.05$, **significant at $p< 0.01$, ns: not statistically significant at $p<0.05$; sample size (N)= 2200

3.1.1 Effect of explant position in the culture media

In order to investigate the effects of explant position on callus initiation rate, bud explants were placed in two different positions as upward and downward directions when culturing in callus initiation media. At the end of one-month incubation period, explants in downward direction showed better response with respect to callus initiation in early stages of the experiment (Figure 3.1). There were 43.6 % more explants with callus initiation when the explants were planted downward vs. upward. This result was supported by high positive correlation (r= 0.44) between first month callus initiation (CI) and position (Table 3.2).

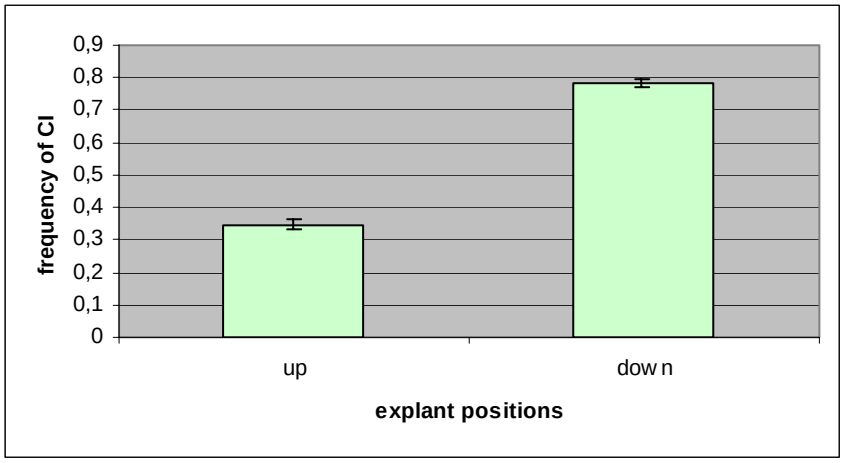


Figure 3.1 Effect of explant position on callus initiation rate at the end of first month incubation (vertical lines on the bar graphs indicated the standard error of estimations)

Although callus initiation rate of the explants in downward direction was significantly higher than that of upward direction at the end of one month, there was no significant difference between two positions for callus initiation rate of the common juniper explants after two months. When calli sizes were considered at the end of two-month incubation period, explants cultured in downward position showed better response by forming larger calli (Figure 3.2). Furthermore, Pearson correlation between callus size and explant position was significant (r=0.20) for the first month of culture, but it was low at the second month of culture (r=0.03) (Table 3.2).

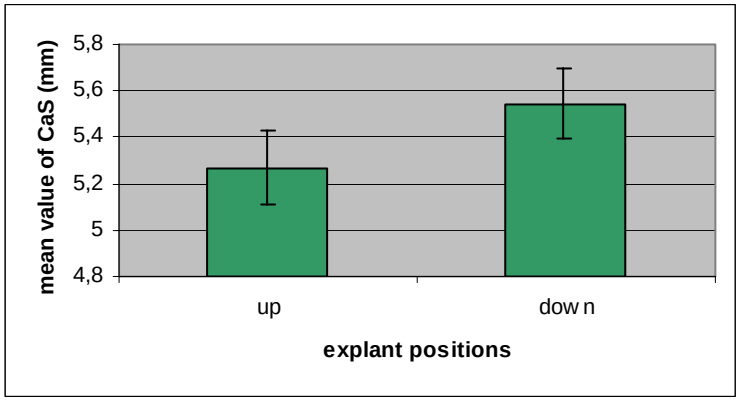


Figure 3.2 Effect of explant position on calli sizes at the end of two-months-culture period (vertical lines on the bar graphs indicated the standard error of estimations)

According to these findings, explant position did not make significant difference on callus initiation rate after the first month of incubation period, although calli sizes were still significantly different. It is also found that two-month incubation was the ideal incubation period for the callus induction of common juniper

regardless of position, before transferring the explants onto organogenesis media.

3.1.2 Effect of genotypes on callus initiation

Throughout the study, explants from four different genotypes were used to determine the effect of genotype on *in vitro* regeneration capacity of common juniper. According to the results of callus induction experiments, genotypes showed significant variation on callus initiation rate (Table 3.1, Figure 3.3). Callus initiation rate ranged from 70% in *Genotype 3 to* 88% in *Genotype 1*.

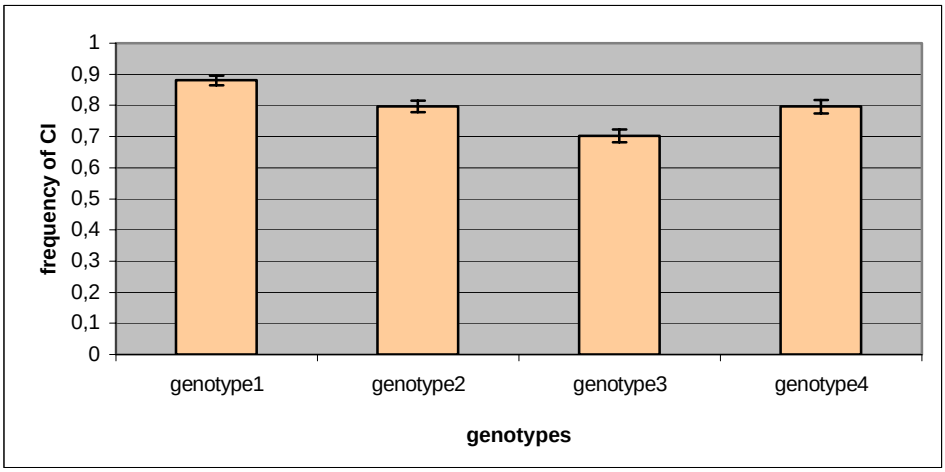


Figure 3.3 Effect of genotype on callus initiation rate at the end of two-month incubation period (vertical lines on the bar graphs indicated the standard error of estimations)

Another parameter used to investigate genotype effect was calli sizes after two-month incubation period. When the mean values of calli sizes were calculated regarding only genotypes in the study, it was found that *Genotype 1* yielded better response (mean calli size=7.68 mm) than others by forming larger calli. *Genotype 4* and *Genotype 2* followed this by producing average calli, 5.5 and 4.8 mm, respectively (Figure 3.4).

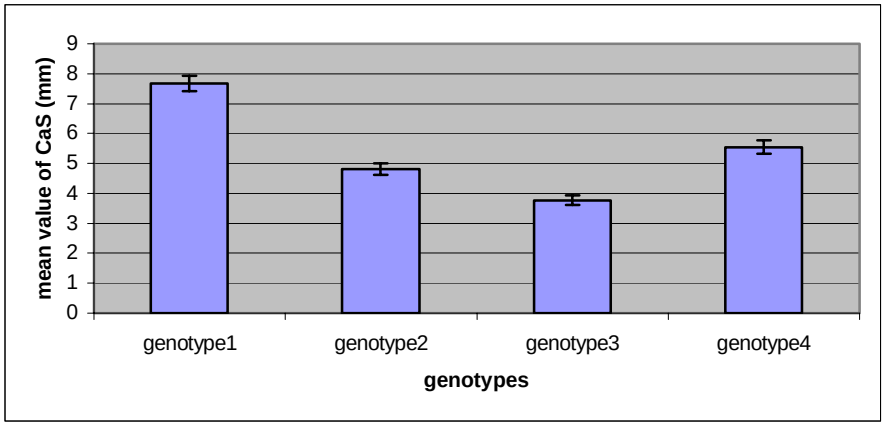


Figure 3.4 Effects of genotype on mean value of calli sizes at the end of two- month-incubation period of callus induction experiments (vertical lines on the bar graphs indicated the standard error of estimations)

Since the variation in responses of different genotypes within species can be enormous, results of micropropagation studies of a species often varies with genotype (Bonga & Aderkas, 1992). According to Confalonieri *et al.* (2003), besides explant type, genotype of the source plant is critical for callus induction in poplar trees. Thus, the findings of this study were consistent with the literature about tissue culture studies in trees.

3.1.3 Effect of gender

Among these four genotypes examined, two of them were female individuals and the others were male ones. Due to the differences among genotypes, gender difference was also investigated for callus formation ability of common juniper. Data on female and male individuals were pooled separately in order to compare the mean values of calli sizes. Mean values obtained from these data were represented in graph shown in Figure 3.5.

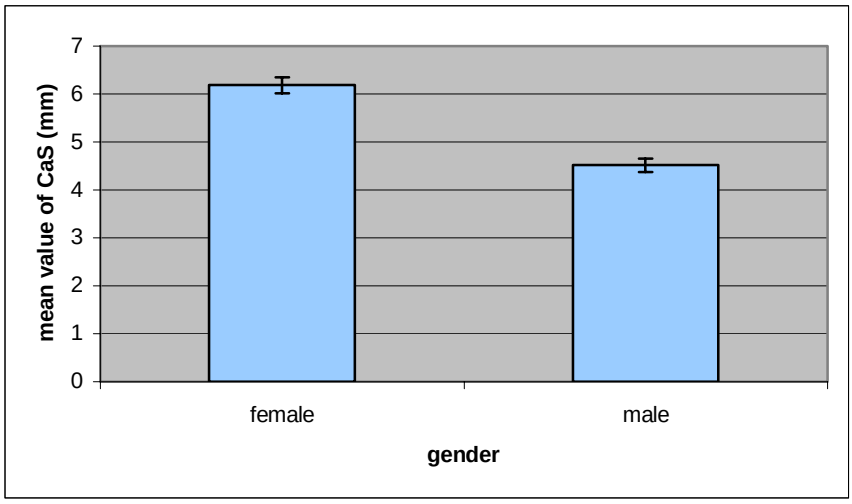


Figure 3.5 Gender difference on calli formation at the end of two-month incubation period of callus induction experiments (vertical lines on the bar graphs indicated the standard error of estimations)

When the mean values of female and male individuals were compared, it was found that gender of an individual, from which explants were obtained, displayed significant difference for the callus formation ability of cultured

explants of common juniper. It was clearly observed that female individuals showed greater callus production response when compared with male ones.

3.1.4 Effect of treatments

In callus induction experiments, 20 treatments including four types of auxins at five different concentrations (Table 2.2) were tested to determine which auxin type at what concentration was effective for the formation of calli. The presence of auxin, which may be combined with a cytokinin, in the nutrient media is required for the callus formation (Confalonieri *et al.*, 2003). These treatments and their effects regarding of calli sizes were shown in Figure 3.6.

When the whole data was considered, the findings of the study indicated that there were significant differences among the treatments applied (Table 3.1). Although it was observed that *Treatments 8, 9,10, 16* and *17* gave better callus initiation, it was hard to clarify which auxin type at which concentration could be the ideal one for callus formation in common juniper explants; since all treatments produced acceptable callus mass (ranging from 2.7 to 7.8 mm). Thus, we investigated the effect of auxin type and concentrations applied separately to decide which auxin type and concentration for best callus formation from common juniper explants could be suggested.

In order to investigate the differences among types of auxins, data of all experimental sets were pooled and mean values of calli sizes for the same auxin group were calculated (Figure 3.7). In order to find the ideal concentration, data of all experimental sets were pooled to calculate mean values of calli sizes for the same concentration group regardless of auxin type. The graphical presentation of the results was given in Figure 3.9.

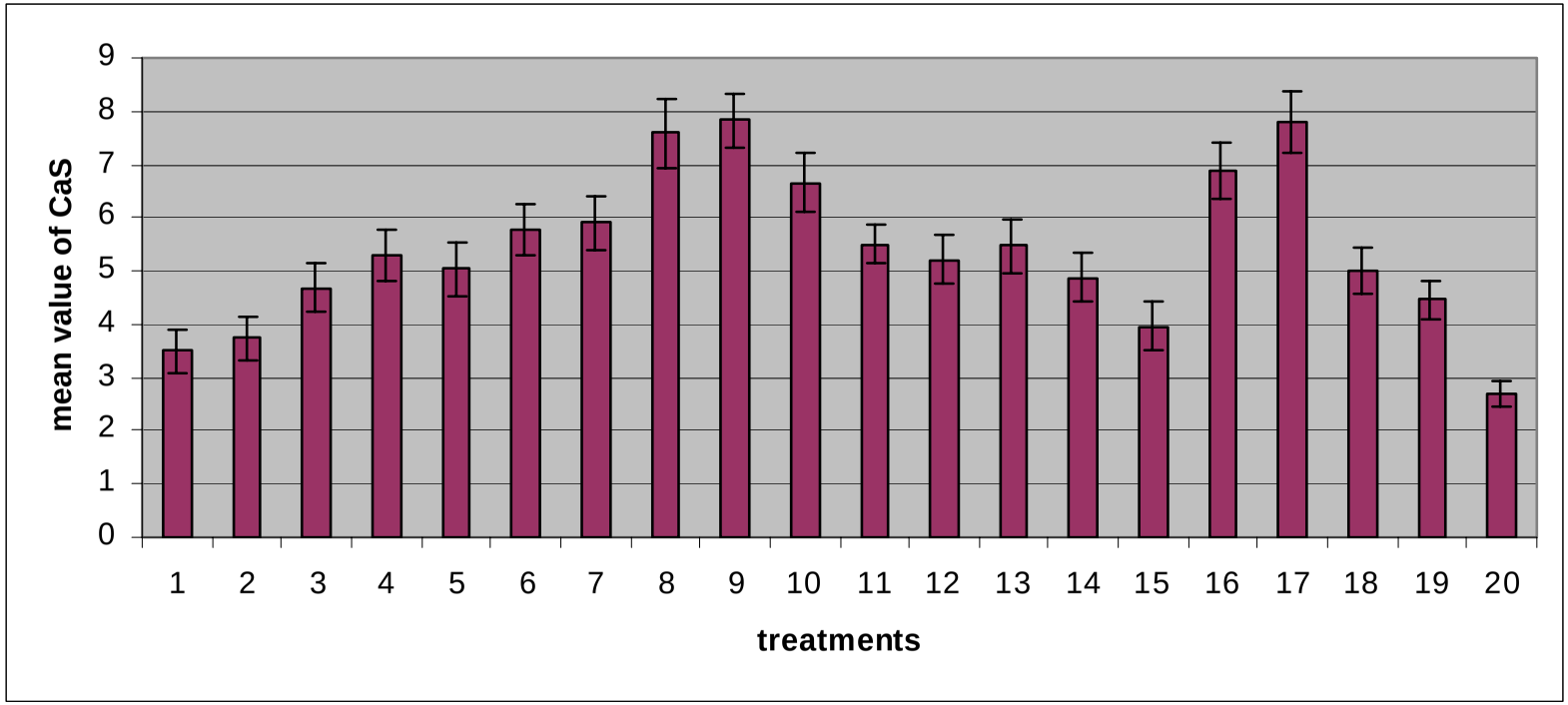


Figure 3.6 Effect of all treatments on callus formation regardless of position and genotype effects. *Treatments 8,9, 10* were 2, 4 10 mg/l IBA and *16 and 17* were 0.5, 1 mg/l 2,4-D, respectively. For the remaining codes, see Table 2.2 (vertical lines on the bar graphs indicated the standard error of estimations)

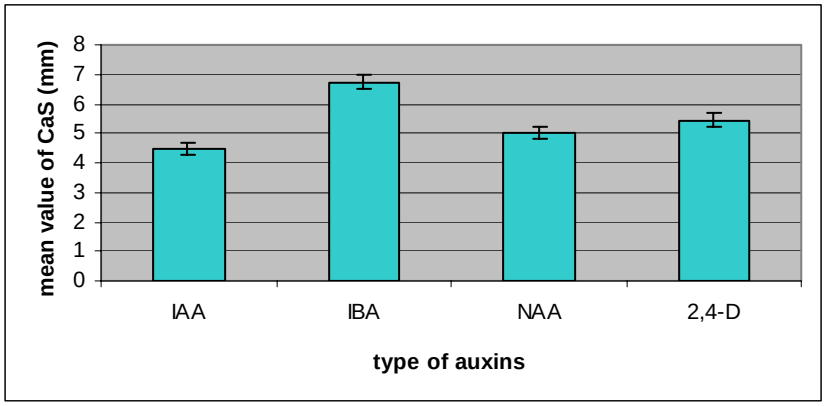


Figure 3.7 Effect of auxin type on callus formation (vertical lines on the bar graphs indicated the standard error of estimations)

Concerning the auxins types, it was clear that explants treated with IBA showed best response by forming larger calli (mean callus size= 6.7 mm). Explants treated with the other auxin types gave similar results on callus formation. Therefore, it was concluded that there was a significant difference between IBA and the other three auxin types. This was not surprise; since according to Nissen & Sutter (1988), although IAA is a natural auxin, about 40% of it in MS medium was destroyed by 20 min autoclaving during media preparation. In addition, iron in the medium promotes the destruction of IAA by light (Dunlap & Robacker, 1988). Our findings showed similar results with the literature, that is, IAA was a poor choice among other auxins types applied. On the other hand, IBA is more advantageous one over other synthetic auxins, since it is metabolized to natural auxin, IAA (Epstein & Lavee, 1984). Besides success with IBA, explants treated with 2,4-D also showed good responses in callus formation. Many reports stated that the phenoxy auxins like 2,4-D are strong promoters of callus induction and growth (Bonga & Aderkas, 1992). Figure 3.8 showed the difference between calli treated with IBA and 2,4-D.

Figure 3.8 Calli induced from the common juniper explants treated with IBA (a) and 2,4-D (b)

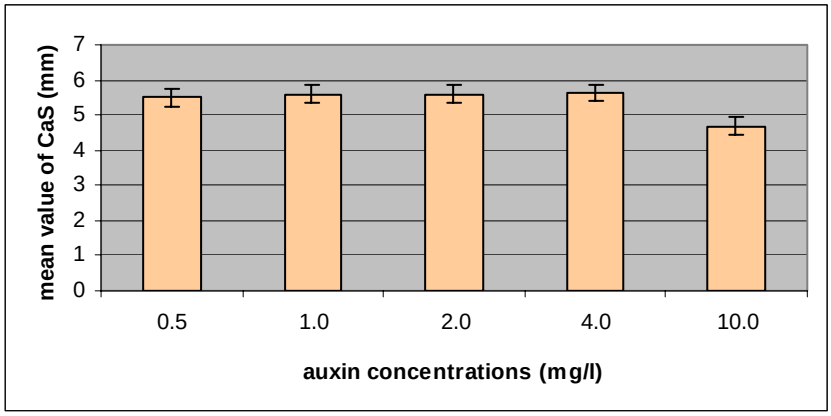


Figure 3.9 Effect of concentration on callus formation (vertical lines on the bar graphs indicated the standard error of estimations)

After deciding the type of auxin, results obtained from different concentrations of pooled data of each set (regardless of auxin type) showed similarity. It appears that first four concentrations of auxin were about equally effective in stimulation of callus production. The mean callus size ranged from 4.7 mm in concentration 10 mg /l of auxin to 5.64 mm in concentration of 4.0 mg/l. However, the concentration of auxin greater than 4.0 mg/l reduced the effect of the auxin on callus production (Figure 3.9).

3.1.5 Effect of explant sampling time

Detailed information about experimental sets was given in Table 2.1. Since they were collected in different seasons, it was a considerable parameter for callus initiation rates of the common juniper explants. First of all, effect of season on survival of the explants was investigated by taking the mean values of alive-explants within each experimental set (Figure 3.10).

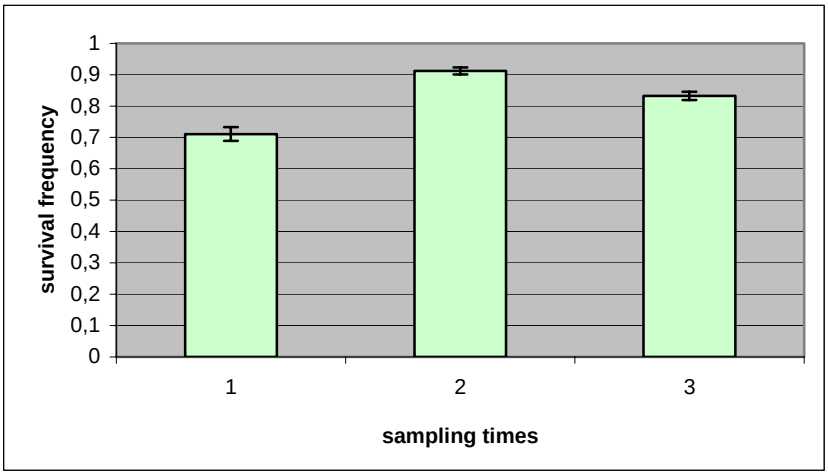


Figure 3.10 Survival rate according to explant sampling times. Sampling times; 1= November 2003; 2= March 2004; and 3= September 2004 (vertical lines on the bar graphs indicated the standard error of estimations)

According to the results, explant sampling done on March, 2004 yielded the best explant survival (91.2%, Figure 3.10). This could be due to high physiological activities of plants at the time prior to their natural flushing times.

When we examined the callus initiation capacity and produced callus size of common juniper explants with respect to sample collection times, again best results were obtained from the explants sampled in the spring of 2004. Callus initiation frequency and callus size were 89.7% and 7.24 mm, whereas other sampling times were less successful for both callus initiation and formation (Figures 3.11 and 3.12).

The largest callus dimension about (7.24 mm) at the end of two-month incubation period was obtained in the sample time of Spring 2004. The other

two sample times gave similar results in the sizes of calli (~4-5mm) (Figure 3.12).

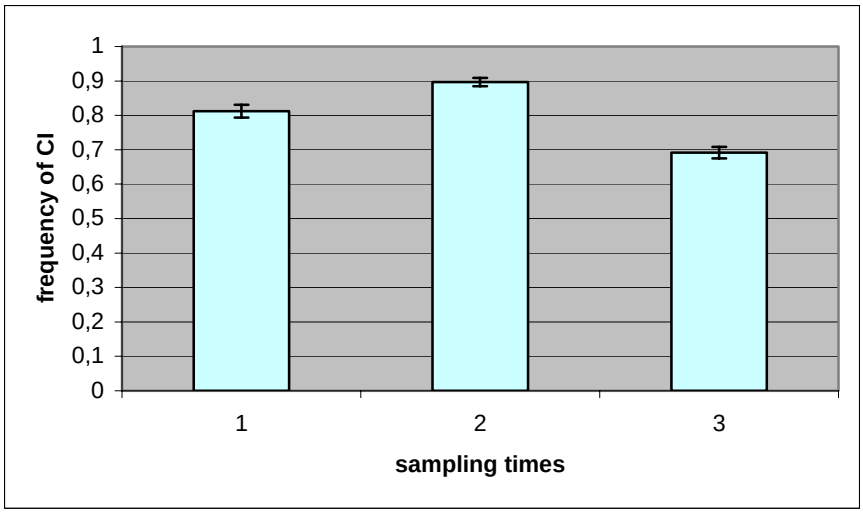


Figure 3.11 Effects of explant sampling times on callus initiation rate of common juniper explants. Sampling times; 1= November 2003; 2= March 2004; and 3= September 2004 (vertical lines on the bar graphs indicated the standard error of estimations)

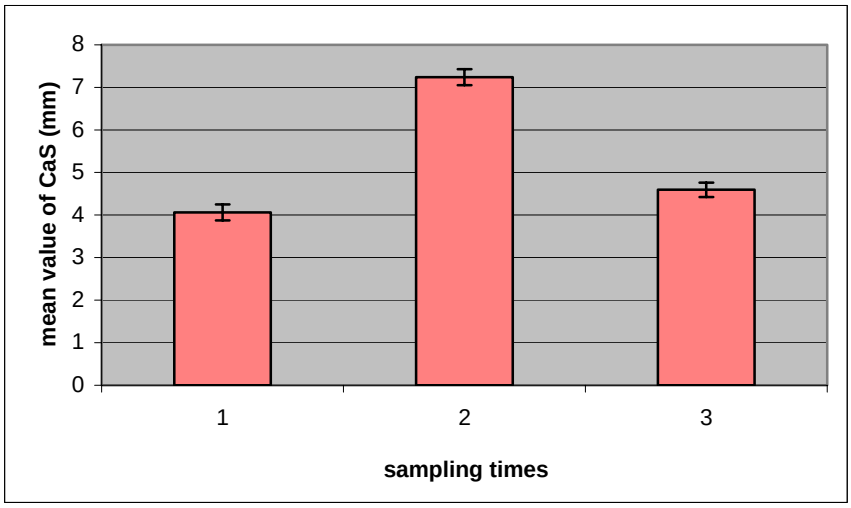


Figure 3.12 Effects of explant sampling times on the development of calli. Sampling times; 1= November 2003; 2= March 2004; and 3= September 2004 (vertical lines on the bar graphs indicated the standard error of estimations)

According to our findings, it was concluded that ideal time for explant sampling time to enhance callus formation capacity of common juniper explants was Spring.

In order to determine which auxin type induced the shoot formation; we compared the pooled data of genotypes within each sampling times (Figure 3.13).

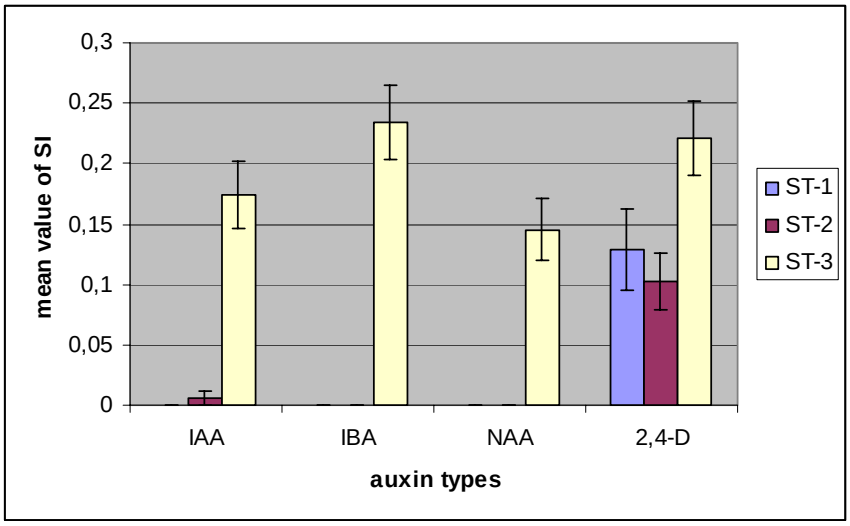


Figure 3.13 Effect of auxin type on shoot induction during the incubation period of callus initiation. ST-1: Sampling time of Nov 2003; ST-2: Sampling time of March 2004; ST-3: Sampling time of Sept 2004 (vertical lines on the bar graphs indicated the standard error of estimations)

As seen from the graph in Figure 3.13, shoot induction was recorded for all types of auxins for the sampling times. However, best results were obtained from the buds treated with IBA and 2,4-D. On the other hand, it was found that 2,4-D was ideal auxin type for shoot induction of common juniper for all sampling times. The high shoot induction of *Sampling Time 3* could be due to the improvements on light conditions of growth room. Previous growth room was illuminated by only four day-length fluorescent lamps (36watt each). Thus, it was concluded that illumination with cool-white fluorescent lamps (36 watts x 2) per each shelf during culturing of the explants was the ideal condition to initiate shoot formation even as they were in the callus induction media. In their review, Confalonieri *et al.* (2003) stated that day length (16/8 h) and high light intensity (5-10 kLux) stimulates the shoot development.

The findings of this study suggest that explant position, genotype and gender of the stock plant, amount of exogeneous growth regulators added in the nutrient medium and sample collection time were critical factors to initiate callus formation in common juniper explants.

3.2 Organogenesis experiments

After deciding the parameters effecting the callus initiation capacity of common juniper, all explants, which transformed into callus or not, were transferred onto 15 different organogenesis treatments (Table 2.4) in order to induce the formation of adventitious buds and shoots. Explants were cultured in these media for a month. Then, they were transferred to the same media excluding the auxin. This part of the study was conducted with two sets at different times (Table 2.1).

In order to understand *in vitro* organogenesis capacity of common juniper; effects of genotype, treatment and explant-sampling time were investigated by dealing with the traits such as amount of green calli (GC), amount of meristemoid formation (MF), number of adventitious buds (NoAB) and adventitious shoot development (ASD) for the first and second months of cultures in organogenesis-treatments. Descriptions and units of these traits were provided in Table 2.5.

For the traits GC, MF, NoAB and ASD for both first and second month cultures, the data were treated as the *Fully Nested ANOVA* and were analyzed by General Linear Model procedures of the Minitab Statistical Software via using adjusted mean squares. The results were given in Table 3.3. Pearson correlation values between these traits were also estimated and the results were presented in Table 3.4.

Table 3.3 ANOVA table for the traits considered in the organogenesis experiments (MS: adjusted mean squares; VC: variance component; df: degrees of freedom)

Traits	Replications df=1	Genotype df=3		Treatment (Genotypes) df=55		Error df=429	
	MS	MS	VC%	MS	VC%	MS	VC%
M1 GC	1.791	2.870	0.84ns	2.373	23.91**	0.656	75.25
M1 MF	84.206	4.975	5.61**	0.971	6.25*	0.613	88.14
M1 NoAB	0.484	0.485	2.40*	0.119	1.65ns	0.139	95.95
M1 ASD	22.241	1.611	2.46*	0.447	0.11ns	0.443	97.43
M2 GC	58.826	24.864	13.10**	4.967	33.50**	0.803	53.40
M2 MF	0.107	13.316	13.70**	1.548	15.90**	0.539	70.40
M2 NoAB	2.881	1.728	3.64*	0.415	3.06ns	0.326	93.30
M2 ASD	30.250	3.660	3.20*	1.027	4.26*	0.744	92.54

*significant at $p< 0.05$; **significant at $p< 0.01$; ns: not statistically significant at $p<0.05$

Table 3.4 Pearson correlation value for the traits considered in organogenesis experiments (see Table 2.5 for the traits descriptions)

Traits	Sampling time	M1-GC	M1-MF	M1-NoAB	M1-ASD	M2-GC	M2-MF	M2-NoAB
M1 GC	-0.197**							
M1 MF	0.295**	0.377**						
M1 NoAB	0.030 ns	0.062ns	0.130**					
M1 ASD	0.227**	0.032 ns	0.254**	0.630**				
M2 GC	-0.273**	0.466**	0.081ns	0.064ns	0.035ns			
M2 MF	0.029 ns	0.273**	0.419**	0.127**	0.117**	0.490**		
M2 NoAB	-0.128**	-0.059 ns	0.192**	0.678**	0.487**	0.078ns	0.129**	
M2 ASD	0.264**	-0.033 ns	0.281**	0.608**	0.766**	0.065ns	0.141**	0.738**

*significant at $p<0.05$; **significant at $p<0.01$; ns: not statistically significant at $p<0.05$; sample size (N)= 630

3.2.1 Effect of genotype

The ANOVA results indicated that there were significant differences among genotypes for organogenesis capacity of the common juniper (Table 3.3). Except for amount of green callus in the first month (M1-GC) trait, genotype effects were significant for all traits and variance components due to genotypes ranged from 2.4 % for the number of adventitious buds in the first month (M1-NoAB) trait to 13.7 % for amount of meristemoid formation in second month (M2-MF) trait (Table 3.3).

Genotypes were significantly different in amount of green alive callus for both months of incubation. Calli from *Genotypes 1* and *2* had significantly higher survival rates than the remaining genotypes. The component of variance due to genotypes in green callus production made up 0.84% and 13.1% of the total variance for first and second month of culture, respectively (Table 3.3, Figure 3.14).

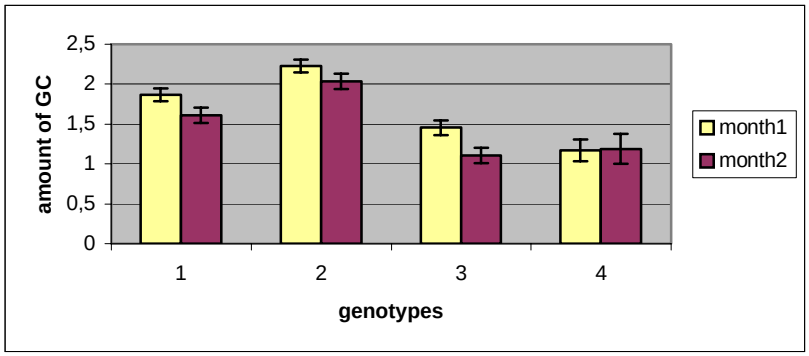


Figure 3.14 Effects of genotype on the amount of alive callus tissues for both first and second month incubation periods in organogenesis treatments (vertical lines on the bar graphs indicated the standard error of estimations)

According to Figure 3.14, it was clear that for the callus formation capacity, even in the organogenesis media, was strongly related to the gender of genotypes. The best green callus production responses were obtained from female individuals (*Genotypes 2 & 1* in Figure 3.14).

Another trait considered was the formation of meristemoids (MF) on calli, since it was reported that increase in the formation of meristemoids could also increase the organ formation. This was supported by obtaining moderate positive correlations between the number of adventitious buds (MI-NoAB, M2-NoAB) and meristemoid formation (M1-MF, M2-MF) for both first (r=0.130) and second (r=0.129) months of incubation (Table 3.4). Data represented in Table 3.3 and Figure 3.15 revealed that there were significant genotypes effects on the amount of meristemoid formation by the explants. The components of variance due to genotypes in amount of meristomoids were 5.6 and 13.7% of total variance for first and second month of culture period (Table 3.3). Calli derived from female genotypes (*Genotypes 1 & 2*) generated high amount of meristemoids again (Figure3.15).

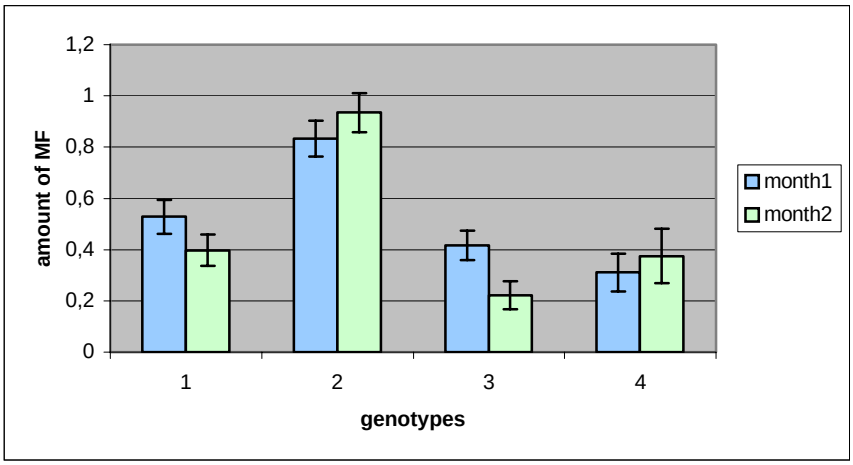


Figure 3.15 Effects of genotype difference on meristemoid formation for both first and second month incubation periods in organogenesis treatments (vertical lines on the bar graphs indicated the standard error of estimations)

When the numbers of adventitious buds formed per each genotype were considered, it was demonstrated that there were significant differences among genotypes (Table 3.3, Figure 3.16). The components of variance due to genotypes in adventitious bud formation were 2.4 and 3.64 % of the total variance for both months of culture, respectively (Table 3.3). *Genotype 2* yielded the highest adventitious bud formation (40.6%) during the first month of incubation. It was decreased to 19.11% at the end of second month, but this was mostly due to the death of calli used as explants (Figure 3.16). Similarly, in a study with *Populus deltoides*, it was demonstrated that genotype was important parameter for *in vitro* formation of adventitious shoots having different physiological requirements (Coleman & Ernst, 1989).

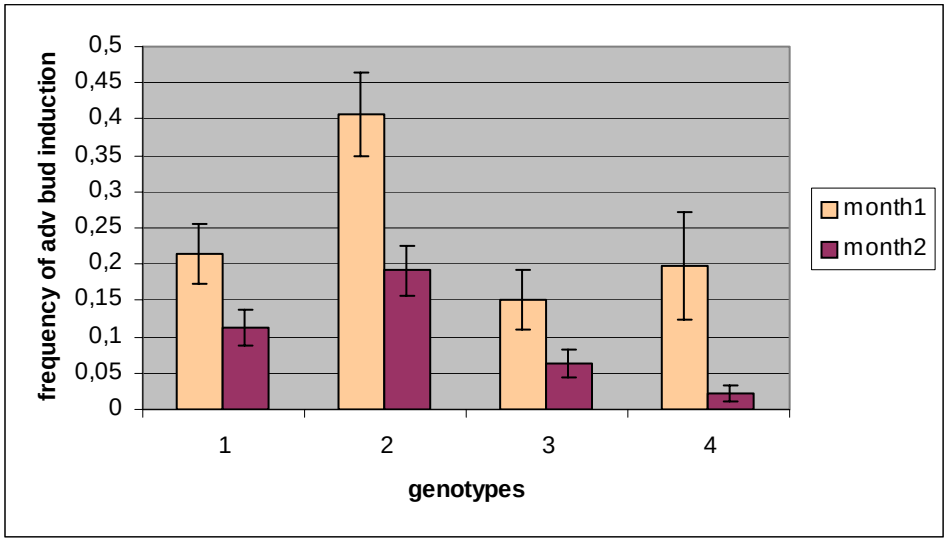


Figure 3.16 Effects of genotypes on the formation of adventitious buds for both first and second month incubation periods in organogenesis treatments (vertical lines on the bar graphs indicated the standard error of estimations)

Finally, the effect of genotypes on the development of adventitious shoots from the adventitious buds used as explants was explored. Genotypes were significantly varied for adventitious shoot development in the first (M1-ASD) and second months of the cultures (M2-ASD) (Figure 3.17). The component of variance due to genotypes in adventitious shoot development varied from 2.46 % in M1-ASD to 3.2 % in M2-ASD (Table 3.3).

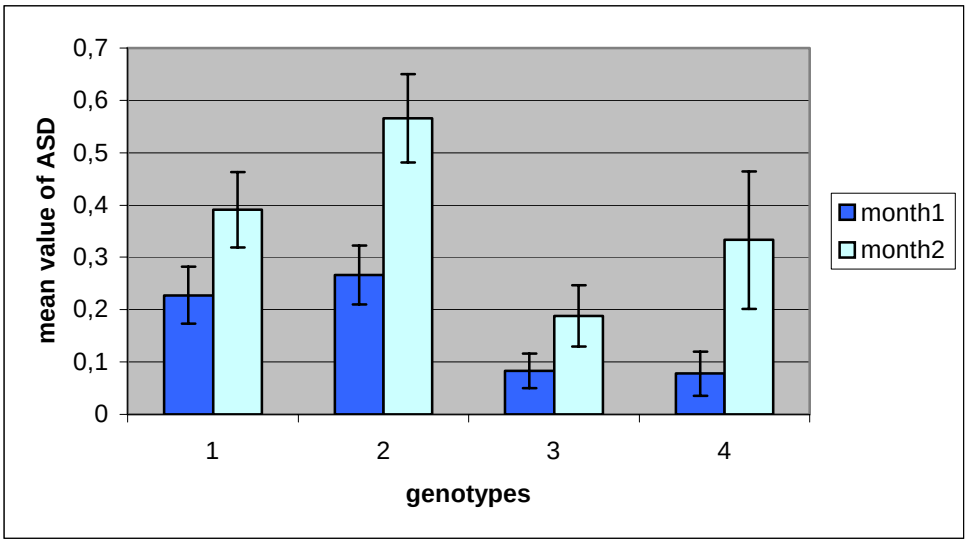


Figure 3.17 Effects of genotype difference on the development of adventitious shoots for both first and second month incubation periods in organogenesis treatments (vertical lines on the bar graphs indicated the standard error of estimations)

Genotype 3 had the lowest adventitious shoot formation compared to others. Thus, it was concluded that after the formation of adventitious buds, genotype had no significant effects on the development of shoots from these buds.

3.2.2 Effect of treatments

In order to investigate the induction of adventitious buds and shoots, 15 different organogenesis treatments including different concentrations of auxin and cytokinin were tested (Table 2.4). During the first month of incubation, explants were cultured in the nutrient media including both 2,4-D and BAP at different concentrations given in Table 2.4. However, after first month of

incubation, 2,4-D components were excluded from the nutrient media while they were refreshed with media only including BAP as the cytokinin.

Treatment effects were significant for all traits except for NoAB and ASD in the first month and NoAB in the second month of culture. The component of total variance due to treatment effects ranged from 0.11 % in M1-ASD to 33.5 % in M2-GC (Table 3.3). Among the applied treatments, *Treatment 6* (0.1mg/l BAP+ 0.5mg/l 2,4-D and 0.1mg/l BAP, respectively) appeared to be the best media for the amount of alive-callus tissue for both months (Figure 3.18). Difference between two months of incubation was resulted from the change of media compositions in a short time. Since the explants did not adapt well to their new culture conditions, they started to secrete polyphenolic compounds in higher amounts when compared with the first month incubation. Thus, these kinds of phenolics led to death of most explants which resulted in the decrease in alive calli. Polyphenolic compounds, which typically accumulate in woody plant tissues, are a critical factor. Because, when tissues are damaged, then these phenolics are oxidized. As a result, the explants are darken, enzymes are inhibited and the explants die (Rout *et al.*, 2000).

The amount of MF with respect to treatments was given in Figure 3.19. The results of the study suggested that of meristemoid formation was similar to that of GC amount as expected. Although the best results were obtained from *Treatment 7* (0.5 mg/l BAP+ 0.5 mg/l 2,4-D) for the first month and from *Treatment 14* (2 mg/l BAP) for the second month, though the standard errors of the estimates were quite high so the difference between *Treatments 7* and *14* may not be real. However, there were number of treatments which could be used for induction of meristemoids in common juniper. Inconsistency among meristemoid formations at different treatments could be related with the phenolic compounds secreted from the explants. Since, these phenolic compounds led to the death of the explants, frequency of meristemoid formation decreased.

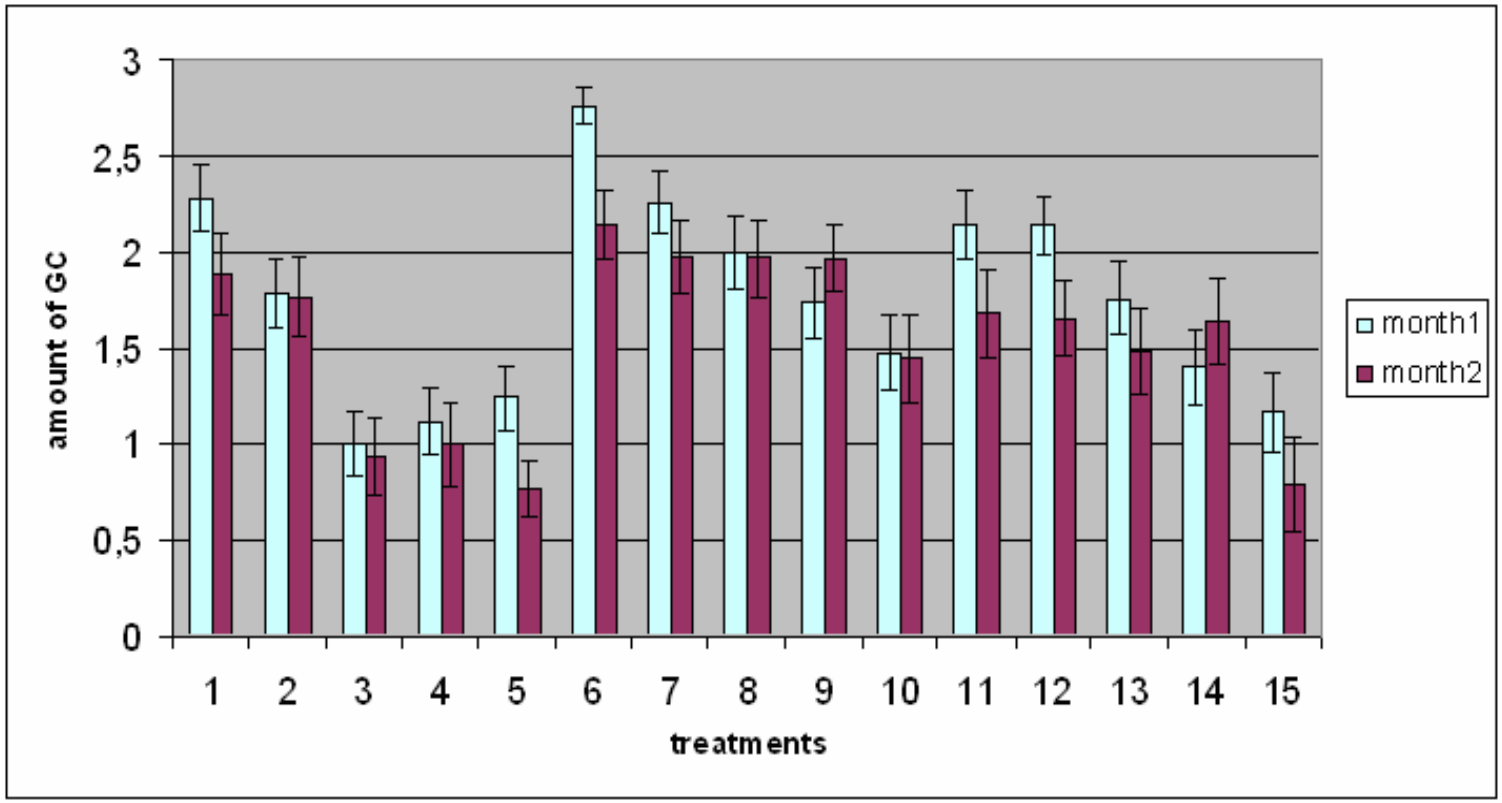


Figure 3.18 Effects of organogenesis treatments on the amount of alive callus tissues for both months of incubation (vertical lines on the bar graphs indicated the standard error of estimations)

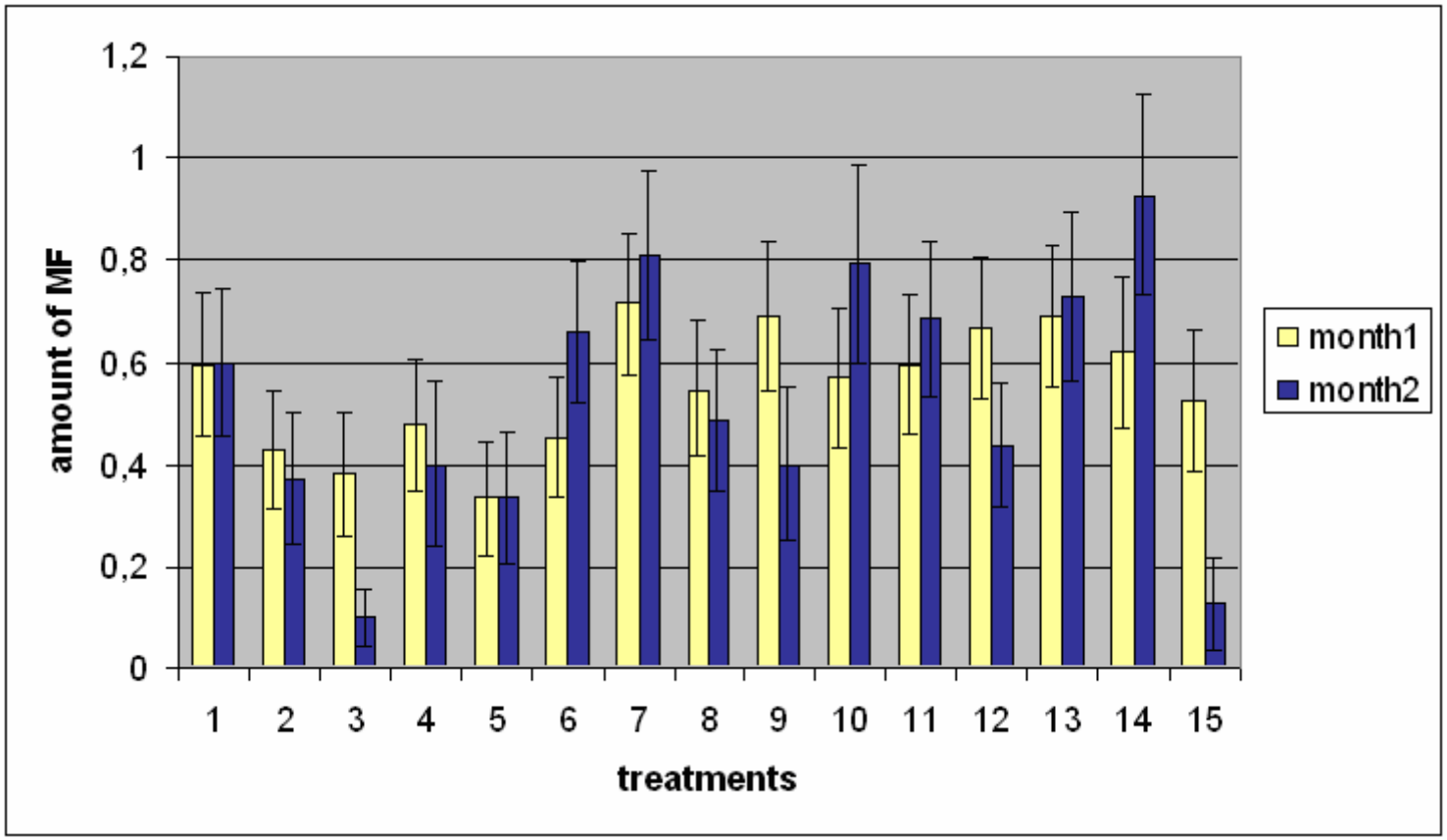


Figure 3.19 Effects of organogenesis treatments on meristemoid formation in the first and second months of cultures (vertical lines on the bar graphs indicated the standard error of estimations)

Since the aim of organogenesis experiment was to initiate adventitious buds and shoots, most important traits considered in this part of the study were the number of adventitious buds and development of the adventitious shoots.

When 2,4-D and BAP were applied together, explants yielded high number of adventitious buds. Best treatments to induce adventitious buds were the *Treatments 11, 9* and *8* for the first month. The frequency of explant with adventitious buds was 18.33, 17.86 and 15.48 % for *Treatments 11, 9,* and *8*, respectively (Figure 3.20). *Treatment 11* was composed of 0.1 mg/l BAP and 1.0 mg/l 2,4-D, while *Treatments 9* and *8* were composed of 0.5 mg/l 2,4-D plus 2 mg/l and 1 mg/l BAP, respectively.

However, at the end of second month of culture, best treatments with induction of adventitious buds were *Treatments 14, 9* and *11* and the frequencies were 47.5, 45.7 and 32.9%, respectively. Figure 3.21 showed the adventitious buds on the alive calli from *Treatments 9* (a) and *14* (b). The *Treatments 14* and *9* were consisted of 2 mg/l BAP while *Treatment 11* contained 0.1 mg/l BAP. This was consistent with the results demonstrated in many studies that high cytokinin concentrations increase the formation of adventitious buds. Confalonieri *et al.* (2003) stated that when cytokinin concentration was lowered, it resulted in the reduction of the frequency of adventitious buds and increase in shoot elongation. From Figure 3.20, it was clearly concluded that adventitious bud formation increased when the auxin component was excluded from the nutrient media, but it was needed in the first stage of the adventitious shoot development in common juniper to increase the yield.

Negussie (1997) stated that explants of the family Cupressaceae responded favorably to relatively low levels of cytokinins. Higher concentration of BAP, especially when combined with high levels of NAA, resulted in inhibition or reduction in the percentage of adventitious bud formation.

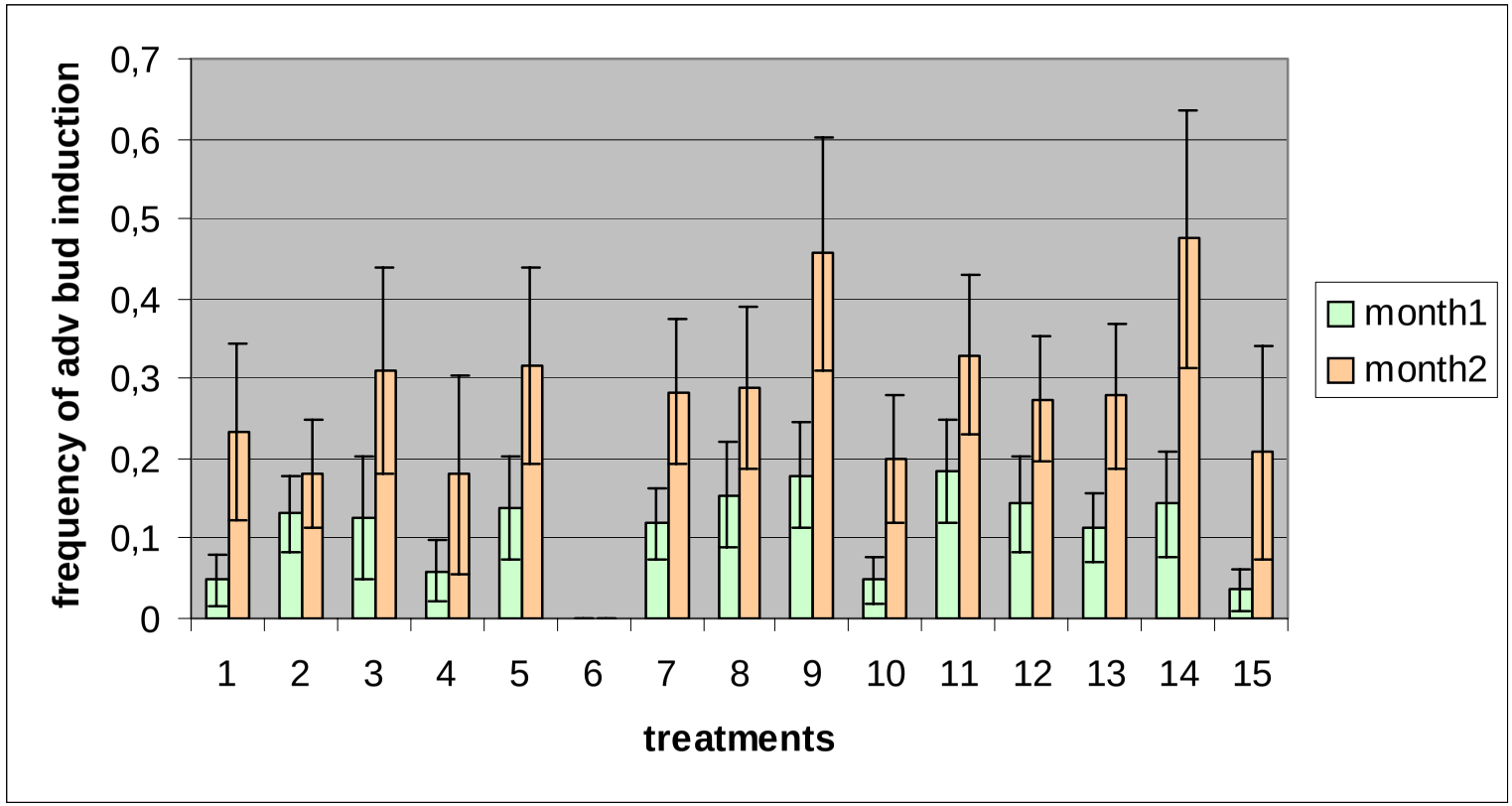


Figure 3.20 Effects of organogenesis treatments on the initiation of adventitious buds for the first and second months of cultures (vertical lines on the bar graphs indicated the standard error of estimations)

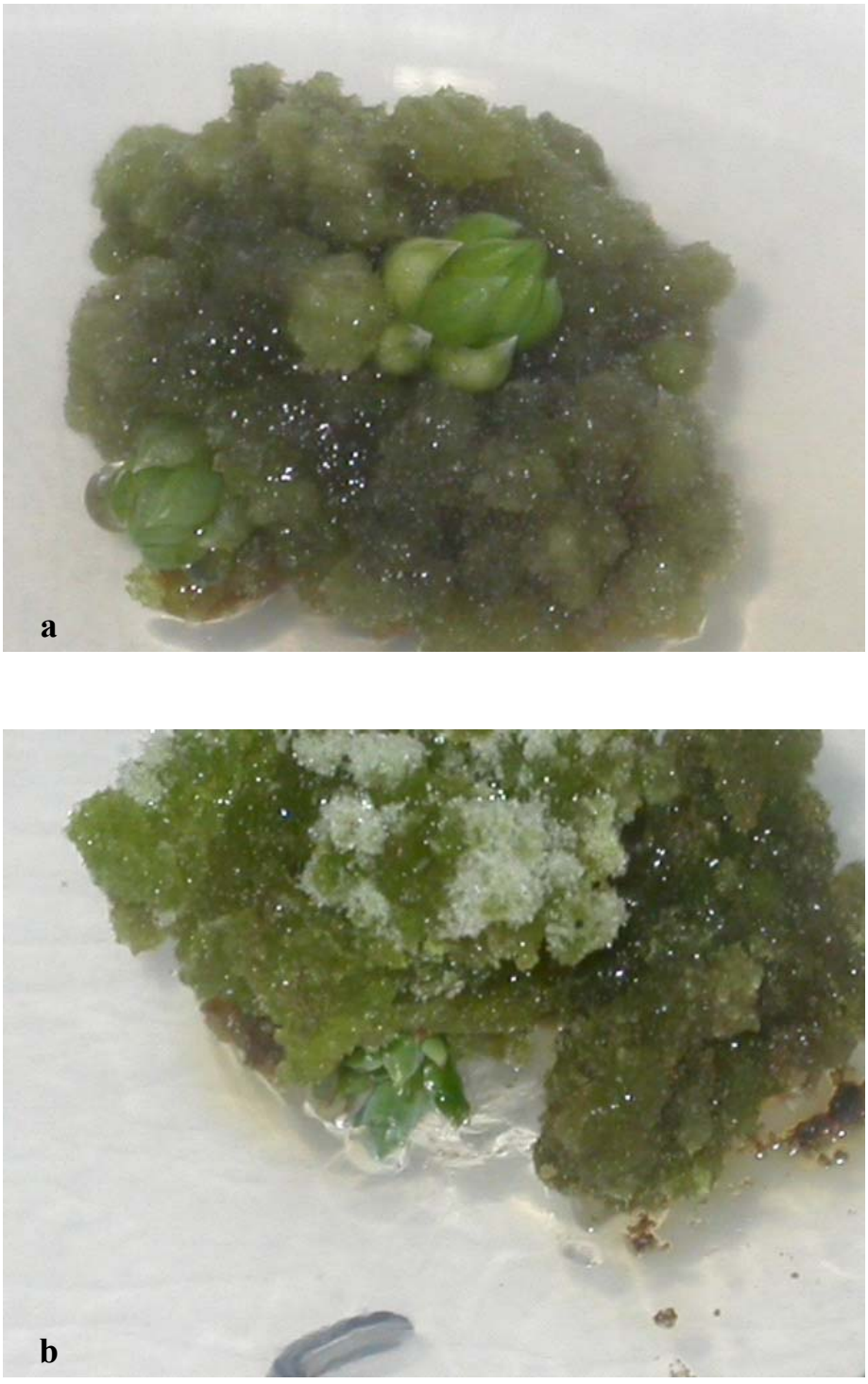


Figure 3.21 Adventitious buds induced from the calli of common juniper explants cultured on a) *Treatment 9* b) *Treatment 14*

In their study, Spanos *et al.* (1997) used benzyladenine in a range of 0.001 mg/l to 1.0 mg/l to increase the numbers of shoots in *Cupressus sempervirens* and *Chamaecyparis lawsoniana*, however, proliferation of shoots were induced without the addition of benzyladenine. This could be due to endogenous synthesis of cytokinin by adventitious shoots established *in vitro*.

Limited number of studies was conducted about the *in vitro* propagation of other juniper species. In their study with *J. oxycedrus*, Gomez & Segura (1995a) found that 1/3 strenght MS media supplemented with different concentrations of BA was ideal for apical explants than nodal explants. They also concluded that nutrient medium and BA concentration affected the length of regenerated shoots.

The development of adventitious shoots was the most critical trait to be considered in organogenesis experiments, since if the adventitious shoots were developed successfully, then the chance of the shoots for adventitious root initiation increased.

Figure 3.22 demonstrated that almost all treatments gave approximate results in the development of adventitious shoots. Although the rate of shoot development increased for much of the treatments in second month, some treatments (*2, 10* and *15*) were unsuccessful. However, these results were not much reliable due to high values of standard error means. Therefore, we concluded that absence of auxin, 2,4-D, in the nutrient media increased the rate of adventitious shoot development especially at high BAP concentrations (1-2 mg/l). Developed shoots from adventitious buds cultured on *Treatments 7* (a) and *11* (b) also given in Figure 3.23.

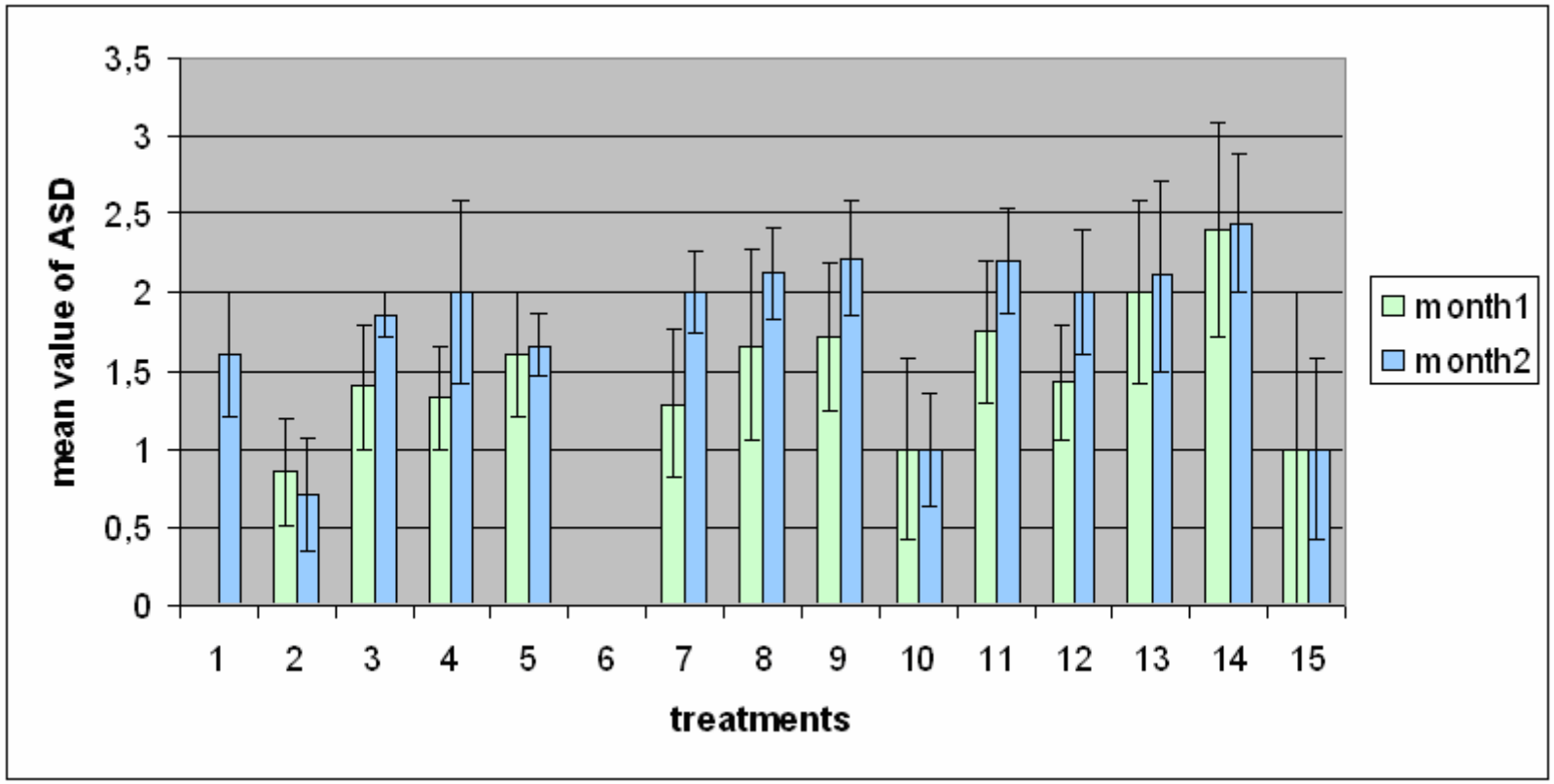


Figure 3.22 Effects of treatments on the mean value of adventitious shoot development (vertical lines on the bar graphs indicated the standard error of estimations)

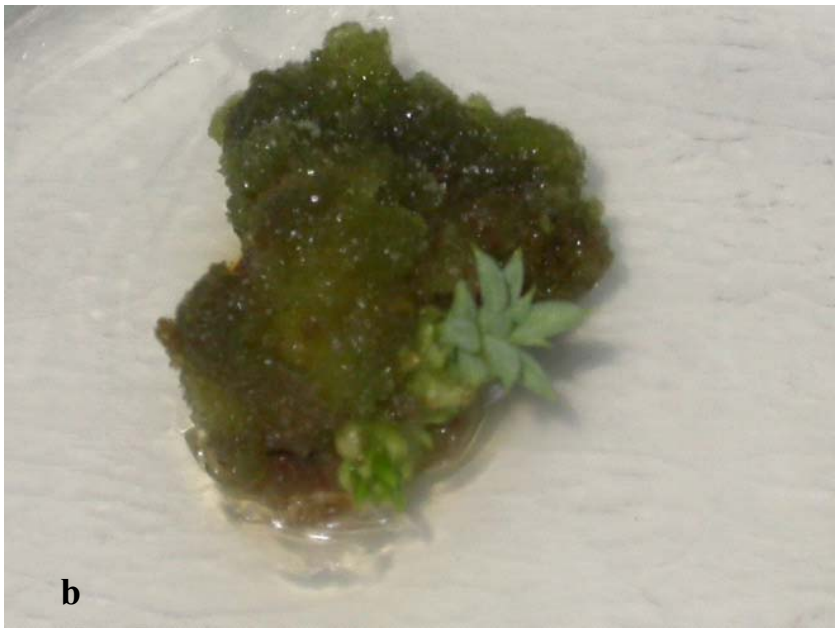


Figure 3.23 Adventitious shoot development on calli cultured on a) *Treatment 7* b) *Treatment 11*

3.2.3 Effect of explant sampling time

The importance of explant sampling times was stated in many reports in the literature. Bonga & Aderkas (1992) stated that conditions for micropropagation of the explants collected from trees in the field were optimal when they were collected in their season.

As opposed to callus induction experiments, survival frequency of GC in the second explant-sampling time (March, 2004) was lower than first explant-sampling time in organogenesis experiment. This could be again due to the death of explants subjected to different nutrient media in a very short time (Figure 3.24), since, graph showed that amount of GC decreased during the second month for both explant-sampling times. Negative correlations (r=-0.197 and r=-0.273, respectively) were found between sampling time and GC amount for both months of cultures in organogenesis experiments, indicating that explants sampled later had lower GC survival values (Table 3.4).

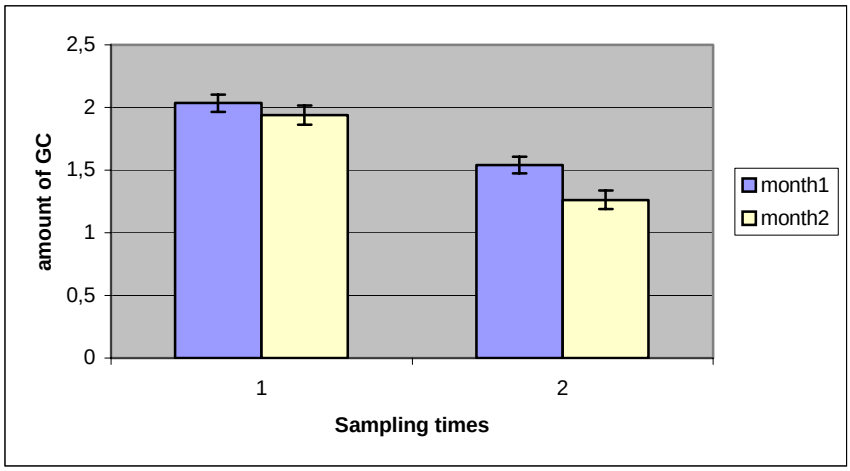


Figure 3.24 Effects of explant sampling times on the amount of green callus. Sampling times; 1= November 2003, 2= March 2004 (vertical lines on the bar graphs indicated the standard error of estimations)

It was observed that meristemoid formation was higher in the second explant-sampling time for the first month. At the end of second month culture, approximate results were obtained from both sampling times (Figure 3.25).

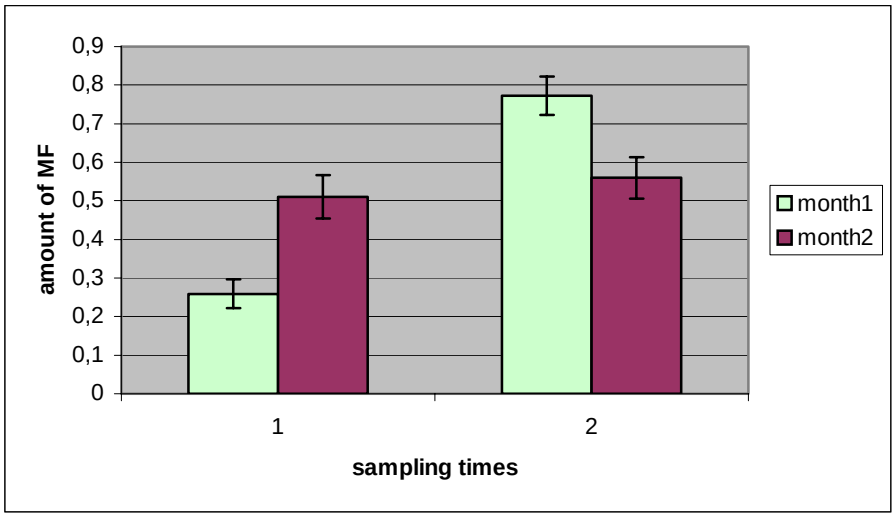


Figure 3.25 Effects of explant sampling times experimental sets on the amount of meristemoid formation. Sampling times; 1= November 2003, 2= March 2004 (vertical lines on the bar graphs indicated the standard error of estimations)

Although the quality of calli decreased in the explants cultured in organogenesis media, necrotic or aged calli continued to induce adventitious bud formation (Figure 3.26). According to data represented in Figure 3.27, there were significant differences between sampling times and for the formation of adventitious buds, the second sampling time with 33.6% success was better than the first sampling time.

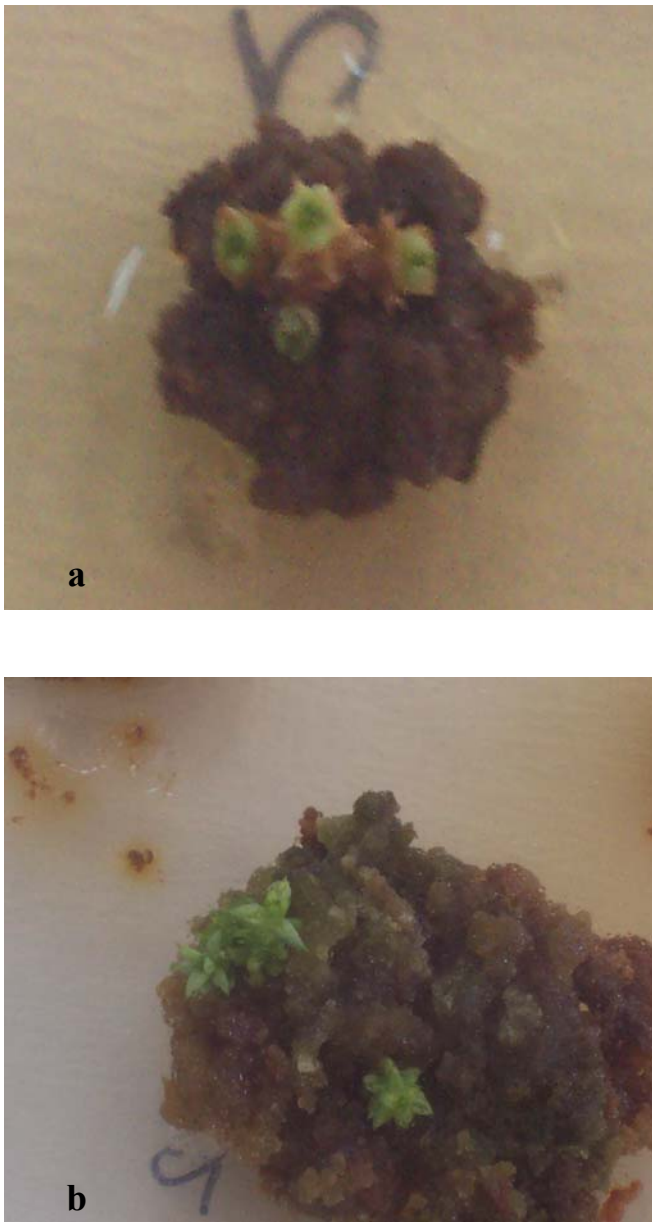


Figure 3.26 Adventitious buds giving shoots on necrotic calli cultured on a) *Treatment 15* b) *Treatment 5*

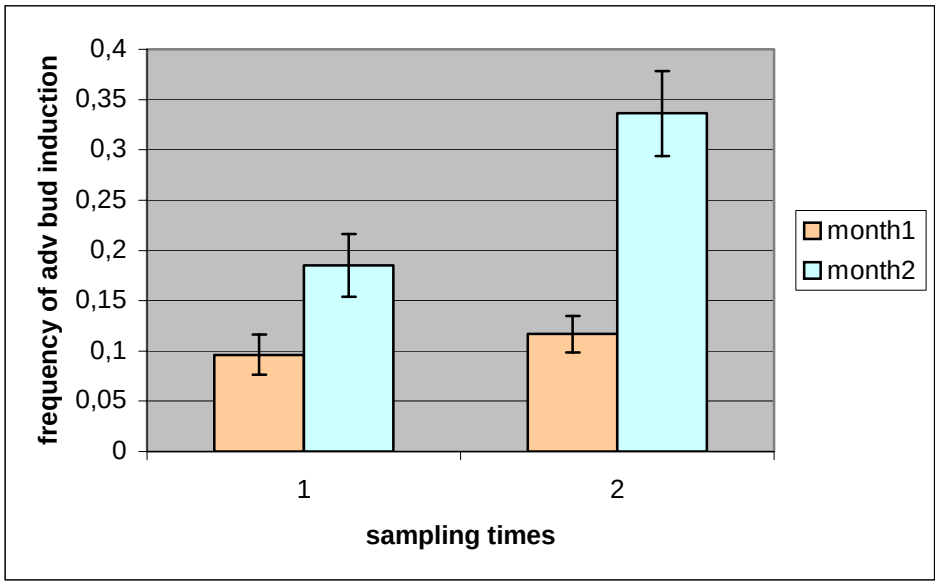


Figure 3.27 Effects of explant sampling times on the formation of adventitious buds. Sampling times; 1= November 2003, 2= March 2004 (vertical lines on the bar graphs indicated the standard error of estimations)

In the micropropagation studies of Sitka spruce (*Picea sitchensis*), it was found that formation of adventitious buds was dependent upon the time of year at which explants were cultured. Maximum adventitious bud formation was obtained at the time prior to their natural flushing time, since buds are naturally forming initials for lateral buds and bud scales, and they are found in a highly active physiological state in this period (Selby & Harvey, 1985).

The development of adventitious shoots was significantly higher in the second experimental set for both months of incubation in organogenesis treatments (Figure 3.28).

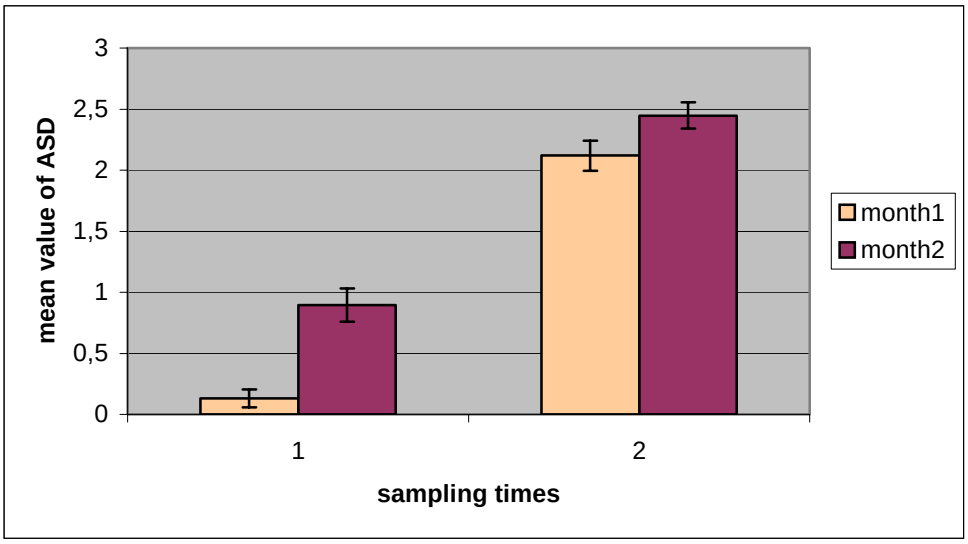


Figure 3.28 Effects of explant sampling times on the development of adventitious shoots. Sampling times; 1= November 2003, 2= March 2004 (vertical lines on the bar graphs indicated the standard error of estimations)

Success in both the number of adventitious buds and their development into shoots in second explant-sampling time mostly related with the incubation conditions which were improved during experiments while the explants from second sampling time were used. In addition, samples were collected before their natural flushing times that agree with the study of Selby and Harvey (1985).

In conclusion, genotypes had a significant effect on all the traits studied throughout the experiment. In addition, after adventitious buds were induced, excluding the auxin from nutrient media resulted in an increase in the number of adventitious buds. Finally, the time of explant collection was important factor for *in vitro* propagation of common juniper.

3.3 Organogenesis Improvement Experiments

From the result of the experiments described in the previous sections and related to organogenesis, it was concluded that genotype had a significant effect on the formation of adventitious buds. However, development of shoots from these buds was not affected by genotype.

It was also demonstrated that explants from female individuals gave best response to initiate adventitious buds in great number than male ones. Explants collected at a time prior to its natural flushing time in the Spring, yielded better results both in the number and in proliferation of adventitious shoots.

Difference between these experiments and previous ones was only increase in the number of treatments applied to optimize the conditions for organogenesis. Organogenesis improvement experiments were also conducted with calli originated from the explants sampled in November 2003 and March 2004 (Table 2.1). While transferring the explants onto improved organogenesis treatments, only calli with adventitious buds and shoots were transferred to improved organogenesis treatments given in Table 2.6. All the adventitious buds and shoots were cultured on adventitious rooting media discussed in the next section.

Because effects of genotype and sample collection time on development of adventitious buds were discussed thoroughly in previous section (section 3.2), here, only the effects of treatments for improved organogenesis were explored. The results of ANOVA by using adjusted mean squares for studied organogenesis related traits and Pearson correlation between traits (GC, MF, NoAB and ASD) were given in Tables 3.5 and 3.6, respectively.

Table 3.5 ANOVA table for the traits considered in the organogenesis improvement experiments (MS: adjusted mean squares; VC: variance component; df: degrees of freedom)

Traits	Replication df=1	Genotype df=3		Treatment df=125		Error df=206	
	MS	MS	VC%	MS	VC%	MS	VC%
M2 GC	58.537	27.563	25.20**	1.472	12.7**	0.961	62.10
M2 MF	4.163	23.722	24.60**	1.238	9.60**	0.897	65.80
M2 NoAB	0.016	0.032	1.42ns	0.018	10.55*	0.014	88.03
M2 ASD	0.002	0.073	1.84ns	0.030	2.68ns	0.033	95.48

*significant at $p<0.05$; **significant at $p<0.01$; ns: not statistically significant at $p<0.05$

Table 3.6 Pearson correlation values for the traits considered in organogenesis improvement experiments (see Table 2.5 for the traits descriptions)

	M2-GC	**M2-MF**	**M2-NoAB**
M2-MF	0.567**		
M2-NoAB	-0.048ns	0.020ns	
M2-ASD	-0.086ns	-0.027ns	0.806**

*significant at $p<0.05$; **significant at $p<0.01$; ns: not statistically significant at $p<0.05$; sample size (N)= 504

3.3.1 Effects of different cytokinins and auxins

To determine whether the type of growth regulators affected the organogenetic capacity of the explants or not, thirty-six different treatments (Table 2.6) which consisted of different cytokinin and auxin types were conducted. BAP, 2-IP and Kinetin as cytokinin types while 2,4-D and IBA as auxin were examined and transferred calli were incubated on these treatments for two months.

Treatments 7-12 and *25-30* appeared to be most suitable ones for survival of green calli (Figure 3.29). *Treatments 7-12* composed of 2-IP and 2,4-D, while *Treatments 25-30* consisted 2-IP and IBA. From these results, it was concluded that 2-IP was the best cytokinin type even for the survival of aged calli (Figure 3.30). Since these calli were 6-month old at the end of incubation period, survival rate of them decreased for many of the treatments. However, it should be pointed out that these treatments also included 0.01% activated charcoal in order to inhibit the negative effects of phenolic compounds on development of callus and organogenesis.

Meristemoid formation data provided in Table 2.5 and Figure 3.31 indicated that there were inconsistent results among the treatments. However, moderate amounts of meristemoid formation on the explants were produced by these treatments at the end of two-month culture period. This was due to the decrease in survival rate and ageing of the explants by this time. Some of treatments including different types of growth regulators induced the formation of meristemoids (Figure 3.31). However, consistent results were not obtained for the treatments including the same type of auxins or cytokinins. Meristemoid formation on calli was displayed in Figure 3.32.

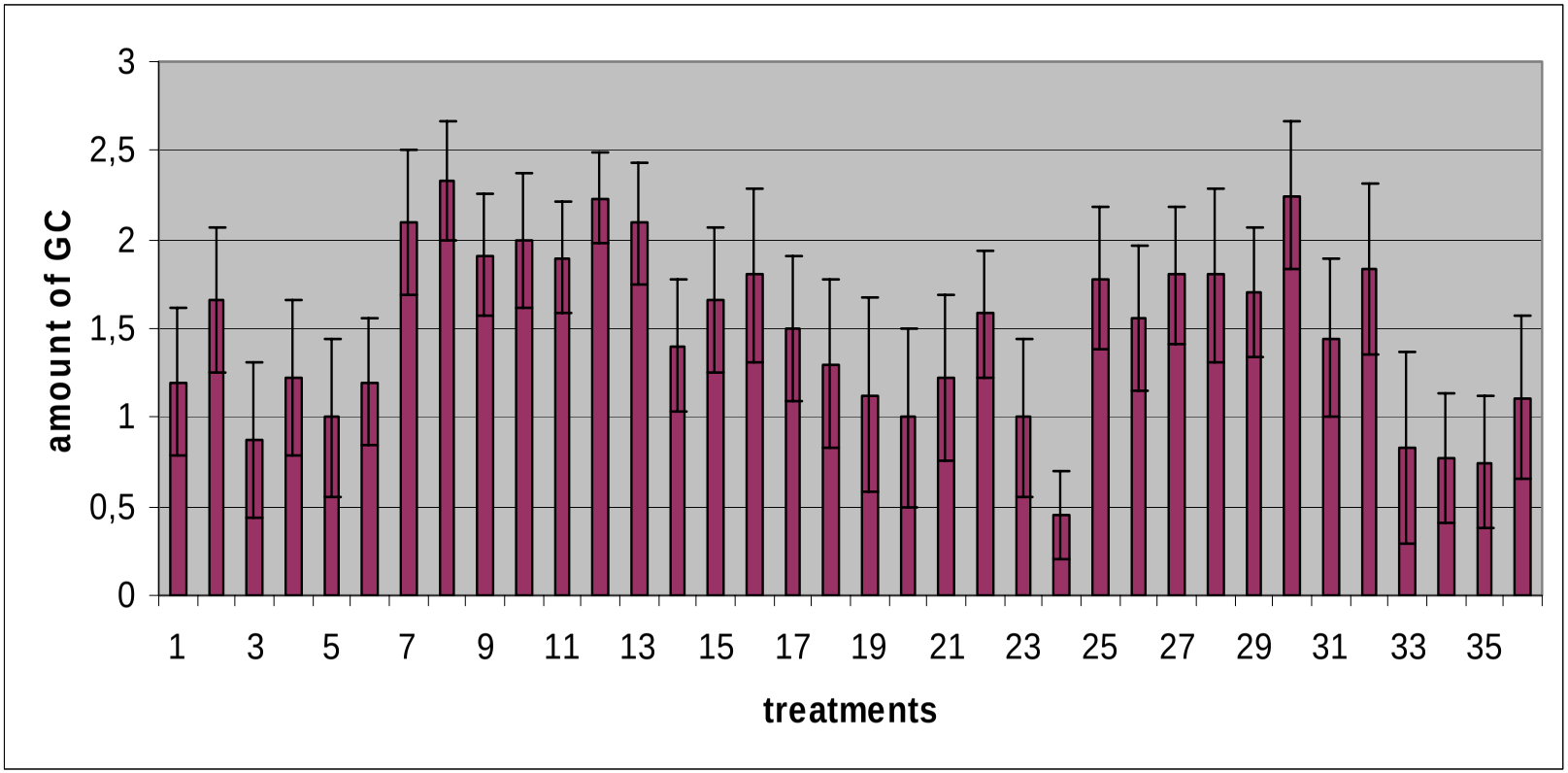


Figure 3.29 Effects of different groups of treatments on the amount of alive callus tissues (vertical lines on the bar graphs indicated the standard error of estimations)

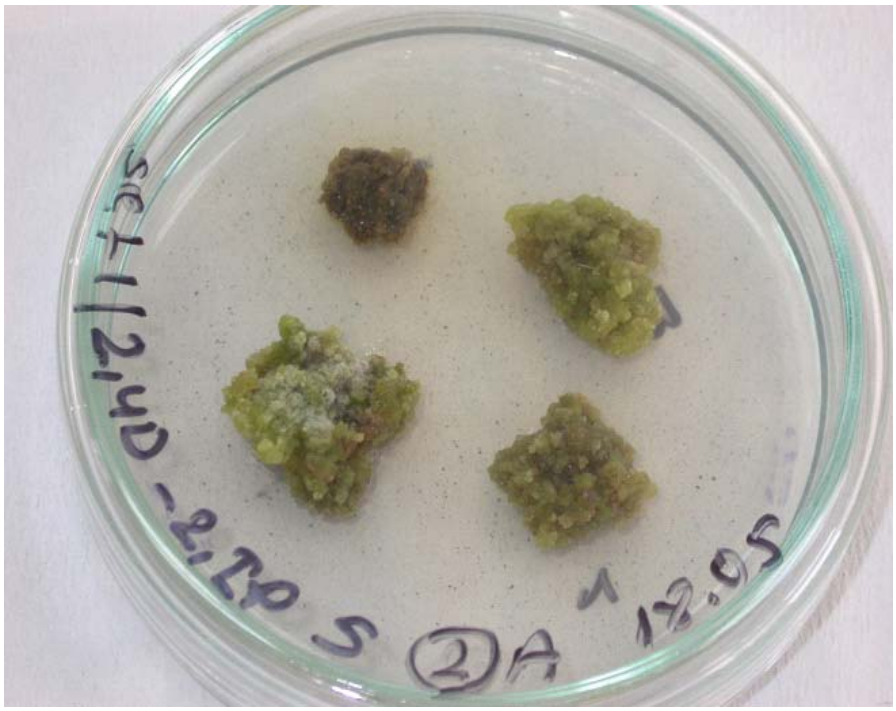


Figure 3.30 Best calli formed by culture media containing 1.0 mg/l 2-IP and 1.0 mg/l 2,4-D

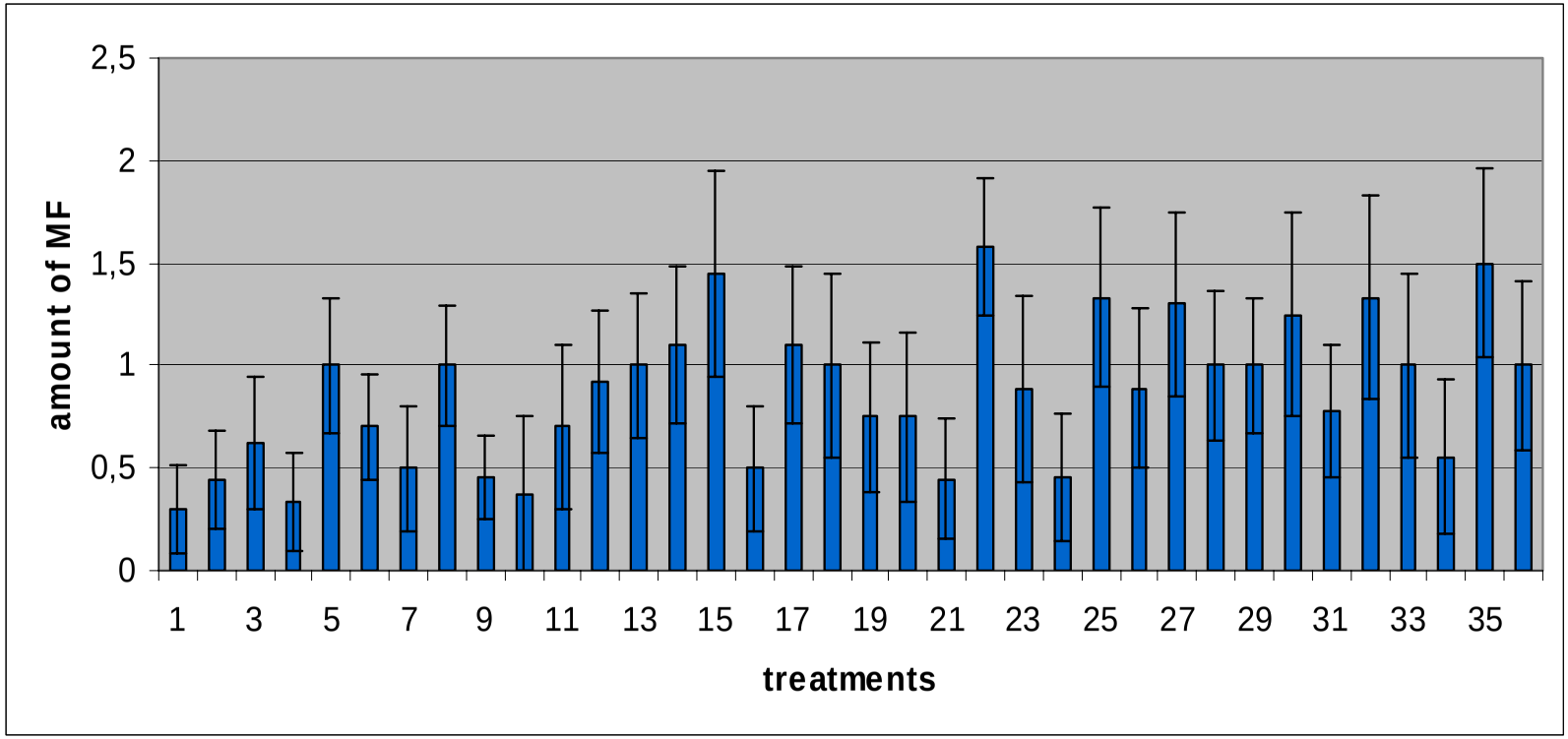


Figure 3.31 Effects of different treatments on the formation of meristemoids (vertical lines on the bar graphs indicated the standard error of estimations)

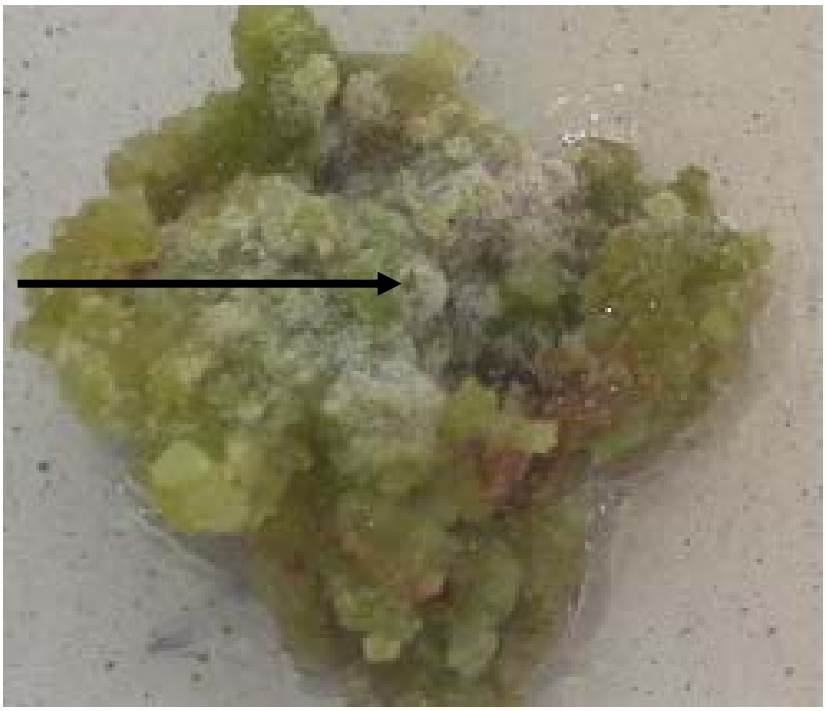


Figure 3.32 Meristemoid formation (pointed with black arrow) on alive tissue mass

Most important traits considered in this section were the formation of adventitious buds and their developments. From the results, it seems that most of the treatments were not suitable for the formation of adventitious buds or due to the ageing of calli at this step. Among thirty-six treatments, only eight of them were suitable for improvement of organogenesis (Figure 3.33). *Treatment 20*, consisted of 1.0 mg/l BAP plus 0.5 mg/l 2,4-D was the best responding one for adventitious bud formation, with the adventitious bud capacity of 37.5%. *Treatment 21* (2.0 mg/l BAP plus 0.5 mg/l IBA) and *Treatment 18* (2.0 mg/l Kinetin plus 1.0 mg/l 2,4-D) followed the *Treatment 20* with 33.3% and 25% bud producing capacities, respectively. The developed adventitious buds were shown in Figure 3.34.

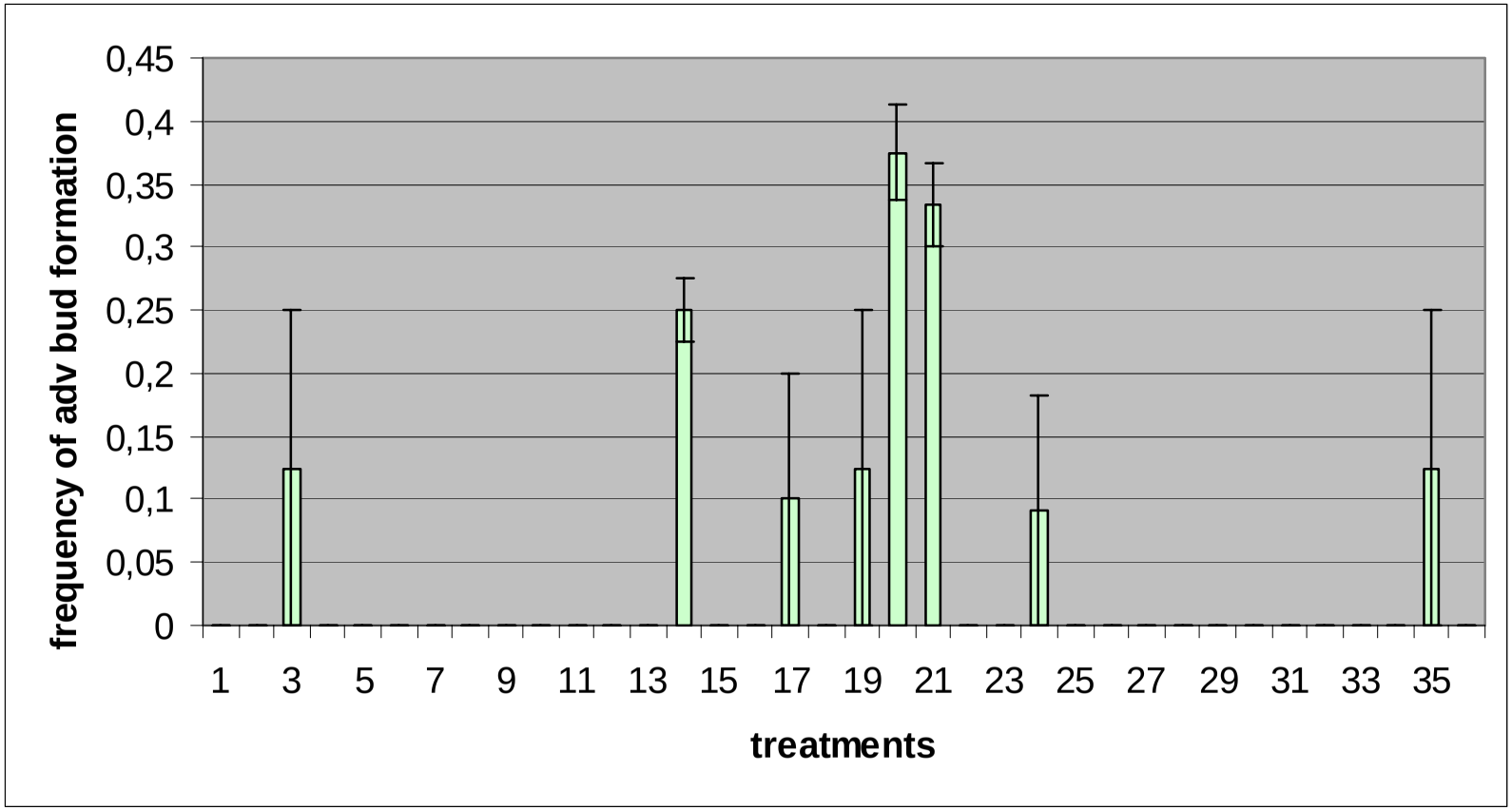


Figure 3.33 Effects of treatments on the formation of adventitious buds (vertical lines on the bar graphs indicated the standard error of estimations)

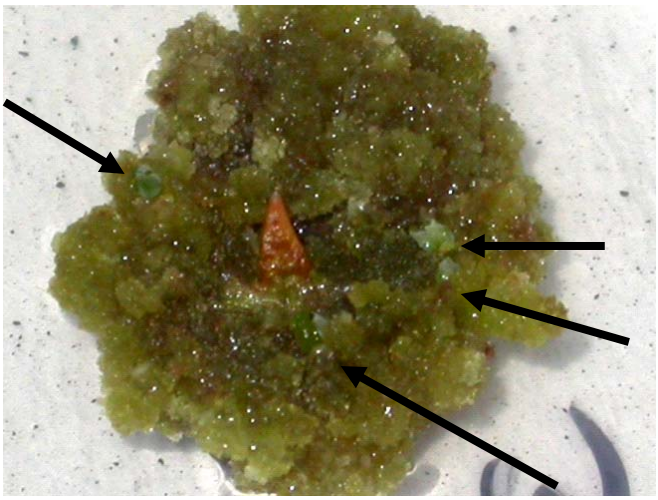


Figure 3.34 Adventitious buds (pointed with black arrows) formed on a green callus

There were no significant differences among the treatments for inducing adventitious shoots from adventitious buds formed through organogenesis improvement experiments (Figure 3.35). Since most of the treatments had no success in the initiation of adventitious buds, data represented in Figure 3.35 were not much reliable, especially due to high standard error means. However, we concluded that composition of the nutrient media did not affect the proliferation of adventitious shoots.

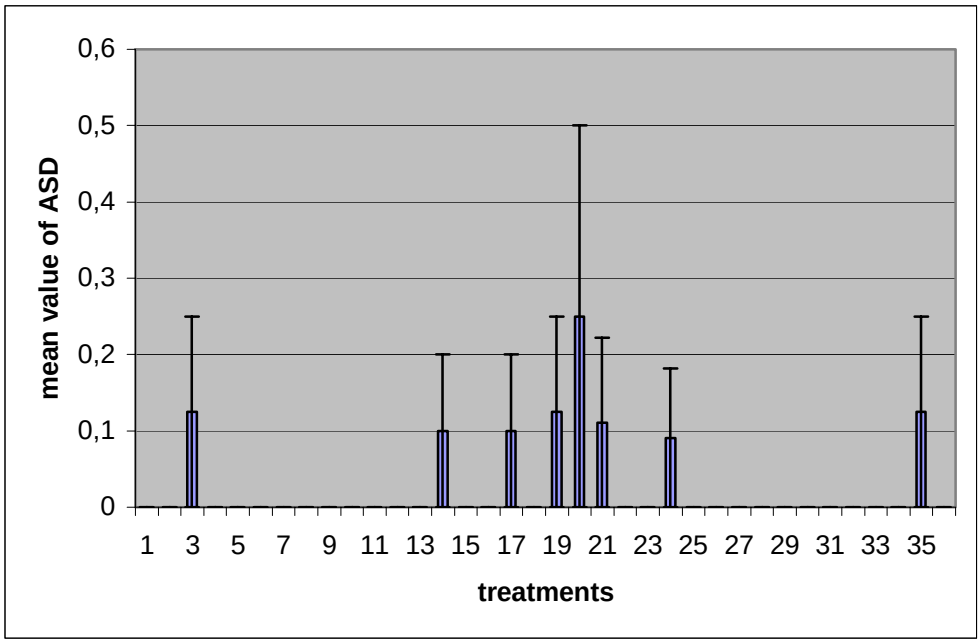


Figure 3.35 Effects of treatments on the development of adventitious shoots (vertical lines on the bar graphs indicated the standard error of estimations)

According to our results, we concluded that age of the calli was the important parameter in the initiation of adventitious buds. It was also found that best treatment for the initiation of adventitious buds from ageing-calli required BAP at a concentration range of 1.0-2.0 mg/l and an auxin at the concentration of 0.5 mg/l regardless of the auxin type used.

Addition of activated charcoal to the nutrient media reduced the production of phenolic compounds. Nevertheless, number of necrotic explants was still high due to ageing, which caused to the reduction of adventitious bud formation.

3.4 Adventitious Rooting Experiments

While calli were transferred to improved organogenesis media, 4-months old shoots at different sizes were cultured in adventitious rooting media. In order to induce root formation in common juniper explants, adventitious shoots were incubated in three different sets of treatments sequentially for different time intervals (section 2.2.2.4). Compositions of these treatments were represented in Table 2.7.

Success was not achieved for the adventitious root induction, and no data was obtained except the survival and development of the adventitious shoots. Two different media (MS & McCown Woody Plant Medium) were used for root induction. Firstly, shoots were cultured in MS based media including IBA at four different concentrations (0-0.005-0.030-0.050 mg/l). Activated charcoal was added to these nutrient media to reduce the effects of excess amount of cytokinin and auxin applied during organogenesis step. Shoots were incubated in these treatments for two months.

At the end of the incubation period, root induction was not observed (Figure 3.36). Most explants were observed to be still alive, and they secreted low amount of phenolic compounds even in the presence of activated charcoal.

Figure 3.36 Adventitious shoots cultured in rooting media

Although root induction was not observed, shoots continued to grow during the incubation in these treatments. Then, these shoots were transferred to half-strength McCown woody plant media supplemented with 0.25 mg/l IBA for a month, and activated charcoal was excluded from the nutrient media while they were refreshed. Whereas most explants were still growing, they started to produce a fragile tissue mass at the cut points. At the end of one-month incubation period, again root induction was not observed. These meristemoid-like structures were removed during the transfer of the adventitious shoots onto the third set of treatments.

Due to the formation of meristemoid-like calli at the cut points of the shoots, IBA concentration was lowered to 0.05 mg/l in the third set of treatment, which included full strength of MC medium. Shoots were treated in this nutrient media for another two-month. Again, no success was obtained for the root induction at

the end of two-month incubation period in the third set of treatment. However, shoots continued to elongate up to 1-1.5 cm in length (Figures 3.37 and 3.38).

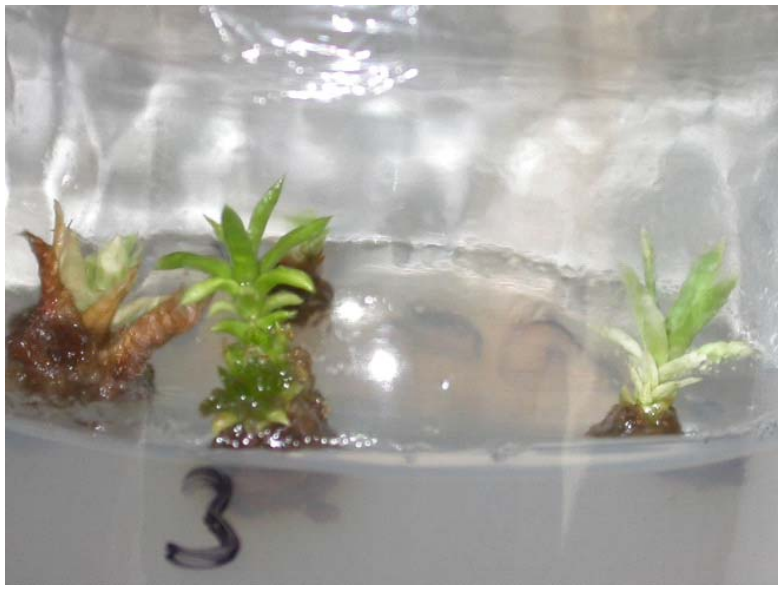


Figure 3.37 Elongating shoots in the rooting media

Figure 3.38 Closer view of elongated adventitious shoots

Although no success was obtained for the adventitious root induction during the incubation periods in these treatments, some undefined structures were obtained while shoots were separated from the calli before culturing them in rooting media (Figure 3.39). These structures were obtained from the explants of second experimental set. These structures could be the elongation of twigs. For root formation and subsequent survival of the plant, elongation of leaves and shoots may be vital. Lambardi *et al.* (1995) stated that as adventitious shoots aged and transfer intervals were increased, rooting occurred spontaneously. However, all treatments which were tested for *in vitro* root induction failed to do so. Junipers are difficult species to induce adventitious roots under *in vitro* conditions. For instance, it was reported that rooting of adventitious shoots proliferated by *in vitro* techniques was also difficult in different species of juniper trees (Gomez & Segura 1995a). They tried to induce adventitious roots in different treatments supplemented with IAA, NAA or IBA, or in combinations of two of them at different concentrations. They concluded that, none of the auxin or auxin combinations gave success in root induction of *Juniperus oxycedrus* leaf explants.

In the studies conducted with *Juniperus excelsa*, Negussie (1997) found that better rooting of the adventitious shoots was obtained by pre-treating them with IBA (1 mg/l) and NAA (0.5 mg/l) or with activated charcoal (1% w/v) for three weeks. Then, these shoots were incubated in a covered seed tray filled with non-sterile compost for four months.

Figure 3.39 Undefined structures elongating from the bottom of adventitious shoots

CHAPTER IV

CONCLUSION

The aim of the study was *in vitro* regeneration of common juniper via indirect organogenesis. Throughout the study, parameters related with the regeneration of common juniper from bud explants of mature individuals were tested. Callus induction, adventitious buds and shoot formation, and rooting capacities of these explants via the method of indirect organogenesis were explored.

In the callus induction experiments, effects of explant position, genotype, gender, treatments and explant sampling time were investigated and the followings could be stated:

- It was clear that although the explant position affected the callus initiation rate during the first month of culture, no significant difference was observed after that time and a two-month callus induction period was adequate time.

- Explant sampling time had significant effect on callus induction from bud explants of common juniper and explant sampling done in the Spring yielded better callus induction from bud explants.

- Genotype of donor plants had a significant effect on callus initiation rate and calli sizes. Also, the gender of the genotypes affected callus induction capacity of explants and explants from female genotypes responded better in callus formation.

- Types of auxin and concentration significantly affected the formation of callus from bud explants and IBA concentration in the range of 0.5-4.0 mg/l was found to be optimum one.

In order to investigate the capacity of common juniper for the formation of adventitious buds and shoots, effects of genotype, treatments and explant sampling time were again tested and following conclusions were drawn:

- Again, genotype of donor plants had a significant effect on induction of adventitious buds from common juniper calli derived from bud explants via indirect organogenesis.

- Adventitious bud induction was increased when auxin component was removed from nutrient media; however, it was needed in the first stage of the adventitious bud formation in common juniper for increasing yield.

- In the absence of auxin in culture media, the rate of adventitious shoot development was improved considerably at high concentrations of BAP (1-2 mg/l).

- To yield great numbers of adventitious buds, the best explant sampling time was found to be the spring season. Explants collected and cultured in Spring season yielded 33.6% higher adventitious buds and 50% adventitious shoot than other sampling times.

- Age of calli had a significant effect on the induction of adventitious buds. It was also found that best treatment for the initiation of adventitious buds even on aged calli was composed of cytokinin BAP at

a concentration range of 1.0-2.0 mg/l and regardless of the type of auxin at the concentration of 0.5 mg/l.

To induce adventitious rooting, series of experiments with various treatments were carried out. Of the treatments tested, none of them was successful for the induction of adventitious rooting of common juniper explants. In the future studies, pre-treatment of adventitious shoots with appropriate auxin type could be suggested before transferring them onto hormone free media.

Although whole plantlet development from callus tissues could not be achieved as indirect organogenesis, however, the results of the study could aid to future studies dealing *in vitro* regeneration and production of secondary chemicals from common juniper.

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APPENDIX A

COMPOSITION OF MURASHIGE & SKOOG (MS) MEDIUM

Table A.1 Composition of MS basal medium (microelements, macroelements, and vitamins)

COMPONENTS	**mg/l**
Micro elements	
$CoCl_2.6H_2O$	0.025
$CuSO_4.5H_2O$	0.025
FeNaEDTA	36.70
H_3BO_3	6.20
KI	0.83
$MnSO_4.H_2O$	16.90
$Na_2MoO_4.2H_2O$	0.25
$ZnSO_4.7H_2O$	8.60
Macro elements	
$CaCl_2$	332.02
KH_2PO_4	170.00
KNO_3	1900.00
$MgSO_4$	180.54
NH_4NO_3	1650.00
Vitamins	
Glycine	2.00
myo-inositol	100.00
Nicotinic acid	0.50
Pyridoxine HCl	0.50
Thiamine HCl	0.10
TOTAL	**4405.19**

APPENDIX B

COMPOSITION OF McCOWN (MC) WOODY PLANT MEDIUM

Table B.1 Composition of MC woody plant medium (microelements, macroelements, and vitamins)

COMPONENTS	**mg/l**
Micro elements	
$CuSO_4.5H_2O$	0.25
FeNaEDTA	36.70
H_3BO_3	6.20
$MnSO_4.H_2O$	22.30
$Na_2MoO_4.2H_2O$	0.25
$ZnSO_4.7H_2O$	8.60
Macro elements	
$CaCl_2$	72.50
$Ca(NO_3)_2.2H_2O$	471.26
KH_2PO_4	170.00
K_2SO_4	990.00
$MgSO_4$	180.54
NH_4NO_3	400.00
Vitamins	
Glycine	2.00
myo-inositol	100.00
Nicotinic acid	0.50
Pyridoxine HCl	0.50
Thiamine HCl	1.00
TOTAL	**2462.60**

APPENDIX C

MEAN VALUES AND STANDARD ERROR MEANS

Table C.1 Trait means, standard errors of mean (SE) and sample size (N) used in the graphs (Figures 3.1 -3.13) of Callus Induction Experiments [a]

Figures	Variable	Mean ± SE	Sample size (N)
		M1-CI [a]	
Figure 3.1	up	0,348 ± 0,015	963
	down	0,784 ± 0,013	996
		M2-CaS [a]	
Figure 3.2	up	5,269 ± 0,159	916
	down	5,545 ± 0,154	938
		M2-CI [a]	
Figure 3.3	genotype 1	0,880 ± 0,015	476
	genotype 2	0,796 ± 0,018	516
	genotype 3	0,701 ± 0,020	499
	genotype 4	0,796 ± 0,021	363
		M2-CaS [a]	
Figure 3.4	genotype 1	7,681 ± 0,260	476
	genotype 2	4,802 ± 0,193	516
	genotype 3	3,768 ± 0,158	499
	genotype 4	5,545 ± 0,229	363
		M2-CaS [a]	
Figure 3.5	female	6,183 ± 0,166	992
	male	4,516 ± 0,136	862

(continued)

		M2-CaS [a]	
Figure 3.6	1	3,494 ± 0,421	83
	2	3,745 ± 0,403	102
	3	4,691 ± 0,443	97
	4	5,293 ± 0,496	99
	5	5,036 ± 0,513	84
	6	5,784 ± 0,488	97
	7	5,898 ± 0,484	98
	8	7,592 ± 0,640	76
	9	7,830 ± 0,509	100
	10	6,663 ± 0,543	98
	11	5,510 ± 0,382	98
	12	5,212 ± 0,455	85
	13	5,465 ± 0,516	86
	14	4,875 ± 0,467	96
	15	3,969 ± 0,451	98
	16	6,871 ± 0,542	101
	17	7,793 ± 0,595	87
	18	5,010 ± 0,430	98
	19	4,453 ± 0,379	95
	20	2,697 ± 0,260	76
		M2-CaS [a]	
Figure 3.7	IAA	4,460 ± 0,206	465
	IBA	6,721 ± 0,238	469
	NAA	4,989 ± 0,204	463
	2,4-D	5,451 ± 0,224	457
		M2-CaS [a]	
Figure 3.9	0,5 mg/l	5,501 ± 0,241	379
	1,0 mg/l	5,594 ± 0,253	372

				(continued)
Figure 3.9	2,0 mg/l		5,583 ± 0,255	357
	4,0 mg/l		5,636 ± 0,242	390
	10,0 mg/l		4,691 ± 0,247	356
			M2-S [a]	
Figure 3.10	ST-1		0,711 ± 0,022	432
	ST-2		0,912 ± 0,011	658
	ST-3		0,832 ± 0,013	764
			M2-CI [a]	
Figure 3.11	ST-1		0,812 ± 0,019	432
	ST-2		0,897 ± 0,012	658
	ST-3		0,691 ± 0,017	764
			M2-CaS [a]	
Figure 3.12	ST-1		4,060 ± 0,189	432
	ST-2		7,242 ± 0,187	658
	ST-3		4,592 ± 0,171	764
			M2-SI [a]	
Figure 3.13	IAA	ST-1	0,000 ± 0,000	189
		ST-2	0,006 ± 0,006	
		ST-3	0,175 ± 0,028	
	IBA	ST-1	0,000 ± 0,000	192
		ST-2	0,000 ± 0,000	
		ST-3	0,234 ± 0,031	
	NAA	ST-1	0,000 ± 0,000	193
		ST-2	0,000 ± 0,000	
		ST-3	0,145 ± 0,025	
	2,4-D	ST-1	0,129 ± 0,033	190
		ST-2	0,102 ± 0,024	
		ST-3	0,221 ± 0,030	

[a] CI: Callus initiation; CaS: Callus size; S: Survival; SI: Shoot induction

Table C.2 Trait means, standard errors of mean (SE) and sample size (N) used in the graphs (Figures 3.14 -3.28) of Organogenesis Experiments

Figures	Variable		Mean ± SE	Sample size (N)
			GC [a]	
Figure 3.14	Genotype 1	M-1	1,867 ± 0,083	180
		M-2	1,609 ± 0,096	151
	Genotype 2	M-1	2,228 ± 0,082	180
		M-2	2,036 ± 0,096	168
	Genotype 3	M-1	1,456 ± 0,094	180
		M-2	1,107 ± 0,094	122
	Genotype 4	M-1	1,167 ± 0,136	90
		M-2	1,188 ± 0,188	48
			MF [a]	
Figure 3.15	Genotype 1	M-1	0,528 ± 0,066	180
		M-2	0,397 ± 0,062	151
	Genotype 2	M-1	0,833 ± 0,069	180
		M-2	0,934 ± 0,076	168
	Genotype 3	M-1	0,417 ± 0,058	180
		M-2	0,221 ± 0,055	122
	Genotype 4	M-1	0,311 ± 0,073	90
		M-2	0,375 ± 0,106	48
			NoAB [a]	
Figure 3.16	Genotype 1	M-1	0,214 ± 0,040	180
		M-2	0,112 ± 0,024	151
	Genotype 2	M-1	0,406 ± 0,057	180
		M-2	0,191 ± 0,034	168

				(continued)
Figure 3.16	Genotype 3	M-1	0,152 ± 0,041	180
		M-2	0,064 ± 0,019	122
	Genotype 4	M-1	0,198 ± 0,074	90
		M-2	0,022 ± 0,011	48
			ASD [a]	
Figure 3.17	Genotype 1	M-1	0,228 ± 0,054	180
		M-2	0,391 ± 0,072	151
	Genotype 2	M-1	0,267 ± 0,056	180
		M-2	0,565 ± 0,084	168
	Genotype 3	M-1	0,083 ± 0,033	180
		M-2	0,188 ± 0,057	122
	Genotype 4	M-1	0,078 ± 0,042	90
		M-2	0,333 ± 0,131	48
			GC [a]	
Figure 3.18	1	M-1	2,286 ± 0,175	42
		M-2	1,886 ± 0,212	35
	2	M-1	1,786 ± 0,182	42
		M-2	1,771 ± 0,205	35
	3	M-1	1,000 ± 0,170	42
		M-2	0,933 ± 0,197	30
	4	M-1	1,119 ± 0,171	42
		M-2	1,000 ± 0,216	25
	5	M-1	1,238 ± 0,170	42
		M-2	0,767 ± 0,149	30
	6	M-1	2,762 ± 0,089	42
		M-2	2,146 ± 0,177	41
	7	M-1	2,262 ± 0,167	42
		M-2	1,973 ± 0,192	37

				(continued)
Figure 3.18	8	M-1	2,000 ± 0,199	42
		M-2	1,970 ± 0,206	33
	9	M-1	1,738 ± 0,193	42
		M-2	1,967 ± 0,176	30
	10	M-1	1,476 ± 0,196	42
		M-2	1,448 ± 0,225	29
	11	M-1	2,143 ± 0,176	42
		M-2	1,684 ± 0,227	38
	12	M-1	2,143 ± 0,151	42
		M-2	1,659 ± 0,193	41
	13	M-1	1,762 ± 0,189	42
		M-2	1,485 ± 0,227	33
	14	M-1	1,405 ± 0,196	42
		M-2	1,643 ± 0,225	28
	15	M-1	1,167 ± 0,201	42
		M-2	0,792 ± 0,241	24
			MF [a]	
Figure 3.19	1	M-1	0,595 ± 0,141	42
		M-2	0,600 ± 0,143	35
	2	M-1	0,429 ± 0,119	42
		M-2	0,371 ± 0,130	35
	3	M-1	0,381 ± 0,123	42
		M-2	0,100 ± 0,056	30
	4	M-1	0,476 ± 0,129	42
		M-2	0,400 ± 0,163	25
	5	M-1	0,333 ± 0,111	42
		M-2	0,333 ± 0,130	30

(continued)

Figure 3.19	6	M-1	0,452 ± 0,119	42
		M-2	0,659 ± 0,138	41
	7	M-1	0,714 ± 0,138	42
		M-2	0,811 ± 0,164	37
	8	M-1	0,548 ± 0,133	42
		M-2	0,485 ± 0,138	33
	9	M-1	0,690 ± 0,147	42
		M-2	0,400 ± 0,149	30
	10	M-1	0,571 ± 0,137	42
		M-2	0,793 ± 0,195	29
	11	M-1	0,595 ± 0,137	42
		M-2	0,684 ± 0,151	38
	12	M-1	0,667 ± 0,139	42
		M-2	0,439 ± 0,121	41
	13	M-1	0,690 ± 0,138	42
		M-2	0,727 ± 0,164	33
	14	M-1	0,619 ± 0,148	42
		M-2	0,929 ± 0,199	28
	15	M-1	0,524 ± 0,137	42
		M-2	0,125 ± 0,091	24
			NoAB [a]	
Figure 3.20	1	M-1	0,048 ± 0,033	42
		M-2	0,234 ± 0,111	35
	2	M-1	0,131 ± 0,048	42
		M-2	0,180 ± 0,068	35
	3	M-1	0,126 ± 0,076	42
		M-2	0,310 ± 0,128	30

(continued)

Figure 3.20	4	M-1	0,059 ± 0,039	42
		M-2	0,180 ± 0,125	25
	5	M-1	0,138 ± 0,064	42
		M-2	0,317 ± 0,123	30
	6	M-1	0,000 ± 0,000	42
		M-2	0,000 ± 0,000	41
	7	M-1	0,119 ± 0,044	42
		M-2	0,284 ± 0,090	37
	8	M-1	0,155 ± 0,065	42
		M-2	0,288 ± 0,102	33
	9	M-1	0,179 ± 0,066	42
		M-2	0,457 ± 0,146	30
	10	M-1	0,048 ± 0,029	42
		M-2	0,200 ± 0,080	29
	11	M-1	0,183 ± 0,064	42
		M-2	0,329 ± 0,100	38
	12	M-1	0,143 ± 0,060	42
		M-2	0,274 ± 0,078	41
	13	M-1	0,114 ± 0,044	42
		M-2	0,279 ± 0,091	33
	14	M-1	0,143 ± 0,065	42
		M-2	0,475 ± 0,162	28
	15	M-1	0,036 ± 0,026	42
		M-2	0,208 ± 0,134	24
			ASD [a]	
Figure 3.22	1	M-1	0,000 ± 0,000	2
		M-2	1,600 ± 0,400	5

(continued)

Figure 3.22	2	M-1	0,857 ± 0,340	7
		M-2	0,714 ± 0,360	7
	3	M-1	1,400 ± 0,400	5
		M-2	1,857 ± 0,143	7
	4	M-1	1,333 ± 0,333	3
		M-2	2,000 ± 0,577	3
	5	M-1	1,600 ± 0,400	5
		M-2	1,667 ± 0,211	6
	6	M-1	0,000 ± 0,000	0
		M-2	0,000 ± 0,000	0
	7	M-1	1,286 ± 0,474	7
		M-2	2,000 ± 0,258	10
	8	M-1	1,667 ± 0,615	6
		M-2	2,125 ± 0,295	8
	9	M-1	1,714 ± 0,474	7
		M-2	2,222 ± 0,364	9
	10	M-1	1,000 ± 0,577	3
		M-2	1,000 ± 0,365	6
	11	M-1	1,750 ± 0,453	8
		M-2	2,200 ± 0,327	10
	12	M-1	1,429 ± 0,369	7
		M-2	2,000 ± 0,405	11
	13	M-1	2,000 ± 0,577	7
		M-2	2,111 ± 0,611	9
	14	M-1	2,400 ± 0,678	5
		M-2	2,444 ± 0,444	9
	15	M-1	1,000 ± 1,000	2
		M-2	1,000 ± 0,577	3

				(continued)
			GC [a]	
Figure 3.24	ST-1	M-1	2,033 ± 0,069	270
		M-2	1,937 ± 0,077	237
	ST-2	M-1	1,542 ± 0,067	360
		M-2	1,262 ± 0,075	252
			MF [a]	
Figure 3.25	ST-1	M-1	0,259 ± 0,037	270
		M-2	0,510 ± 0,057	237
	ST-2	M-1	0,772 ± 0,050	360
		M-2	0,559 ± 0,053	252
			NoAB [a]	
Figure 3.27	ST-1	M-1	0,096 ± 0,020	270
		M-2	0,185 ± 0,031	237
	ST-2	M-1	0,117 ± 0,018	360
		M-2	0,336 ± 0,042	252
			ASD [a]	
Figure 3.28	ST-1	M-1	0,130 ± 0,072	23
		M-2	0,895 ± 0,135	38
	ST-2	M-1	2,118 ± 0,121	51
		M-2	2,446 ± 0,107	65

[a] GC: Alive callus tissue; MF: Meristemoid formation; NoAB: Number of adventitious buds; ASD; Adventitious shoot development

APPENDIX C (continued)

Table C.3 Trait means, standard errors of mean (SE) and sample size (N) used in the graphs (Figures 3.29 -3.35) of Organogenesis Improvement Experiments

Figures	Variable	Mean ± SE	Sample size (N)
		M2-GC [a]	
Figure 3.29	1	1,200 ± 0,416	10
	2	1,667 ± 0,408	9
	3	0,875 ± 0,441	8
	4	1,222 ± 0,434	9
	5	1,000 ± 0,441	9
	6	1,200 ± 0,359	10
	7	2,100 ± 0,407	10
	8	2,333 ± 0,333	9
	9	1,909 ± 0,343	11
	10	2,000 ± 0,378	8
	11	1,900 ± 0,314	10
	12	2,231 ± 0,257	13
	13	2,091 ± 0,343	11
	14	1,400 ± 0,371	10
	15	1,667 ± 0,408	9
	16	1,800 ± 0,490	10
	17	1,500 ± 0,401	10
	18	1,300 ± 0,473	10
	19	1,125 ± 0,549	8
	20	1,000 ± 0,500	8
	21	1,222 ± 0,465	9

			(continued)
Figure 3.29	22	1,583 ± 0,358	12
	23	1,000 ± 0,441	9
	24	0,455 ± 0,247	11
	25	1,778 ± 0,401	9
	26	1,556 ± 0,412	9
	27	1,800 ± 0,389	10
	28	1,800 ± 0,490	10
	29	1,700 ± 0,367	10
	30	2,250 ± 0,412	8
	31	1,444 ± 0,444	9
	32	1,833 ± 0,477	6
	33	0,833 ± 0,543	6
	34	0,778 ± 0,364	9
	35	0,750 ± 0,366	8
	36	1,111 ± 0,455	9
		M2-MF [a]	
Figure 3.31	1	0,300 ± 0,213	10
	2	0,444 ± 0,242	9
	3	0,625 ± 0,324	8
	4	0,333 ± 0,236	9
	5	1,000 ± 0,333	9
	6	0,700 ± 0,260	10
	7	0,500 ± 0,307	10
	8	1,000 ± 0,289	9
	9	0,455 ± 0,207	11
	10	0,375 ± 0,375	8
	11	0,700 ± 0,396	10
	12	0,923 ± 0,348	13

			(continued)
Figure 3.31	13	1,000 ± 0,357	11
	14	1,100 ± 0,379	10
	15	1,444 ± 0,503	9
	16	0,500 ± 0,307	10
	17	1,100 ± 0,379	10
	18	1,000 ± 0,447	10
	19	0,750 ± 0,366	8
	20	0,750 ± 0,412	8
	21	0,444 ± 0,294	9
	22	1,583 ± 0,336	12
	23	0,889 ± 0,455	9
	24	0,455 ± 0,312	11
	25	1,333 ± 0,441	9
	26	0,889 ± 0,389	9
	27	1,300 ± 0,448	10
	28	1,000 ± 0,365	10
	29	1,000 ± 0,333	10
	30	1,250 ± 0,491	8
	31	0,778 ± 0,324	9
	32	1,333 ± 0,494	6
	33	1,000 ± 0,447	6
	34	0,556 ± 0,377	9
	35	1,500 ± 0,463	8
	36	1,000 ± 0,408	9
		NoAB [a]	
Figure 3.33	1	0,000 ± 0,000	10
	2	0,000 ± 0,000	9
	3	0,125 ± 0,125	8

			(continued)
Figure 3.33	4	0,000 ± 0,000	9
	5	0,000 ± 0,000	9
	6	0,000 ± 0,000	10
	7	0,000 ± 0,000	10
	8	0,000 ± 0,000	9
	9	0,000 ± 0,000	11
	10	0,000 ± 0,000	8
	11	0,000 ± 0,000	10
	12	0,000 ± 0,000	13
	13	0,000 ± 0,000	11
	14	0,250 ± 0,025	10
	15	0,000 ± 0,000	9
	16	0,000 ± 0,000	10
	17	0,100 ± 0,100	10
	18	0,000 ± 0,000	10
	19	0,125 ± 0,125	8
	20	0,375 ± 0,037	8
	21	0,333 ± 0,033	9
	22	0,000 ± 0,000	12
	23	0,000 ± 0,000	9
	24	0,091 ± 0,091	11
	25	0,000 ± 0,000	9
	26	0,000 ± 0,000	9
	27	0,000 ± 0,000	10
	28	0,000 ± 0,000	10
	29	0,000 ± 0,000	10
	30	0,000 ± 0,000	8
	31	0,000 ± 0,000	9

			(continued)
Figure 3.33	32	0,000 ± 0,000	6
	33	0,000 ± 0,000	6
	34	0,000 ± 0,000	9
	35	0,125 ± 0,125	8
	36	0,000 ± 0,000	9
		ASD [a]	
Figure 3.35	1	0,000 ± 0,000	10
	2	0,000 ± 0,000	9
	3	0,125 ± 0,125	8
	4	0,000 ± 0,000	9
	5	0,000 ± 0,000	9
	6	0,000 ± 0,000	10
	7	0,000 ± 0,000	10
	8	0,000 ± 0,000	9
	9	0,000 ± 0,000	11
	10	0,000 ± 0,000	8
	11	0,000 ± 0,000	10
	12	0,000 ± 0,000	13
	13	0,000 ± 0,000	11
	14	0,100 ± 0,100	10
	15	0,000 ± 0,000	9
	16	0,000 ± 0,000	10
	17	0,100 ± 0,100	10
	18	0,000 ± 0,000	10
	19	0,125 ± 0,125	8
	20	0,250 ± 0,250	8
	21	0,111 ± 0,111	9
	22	0,000 ± 0,000	12

			(continued)
Figure 3.35	23	0,000 ± 0,000	9
	24	0,091 ± 0,091	11
	25	0,000 ± 0,000	9
	26	0,000 ± 0,000	9
	27	0,000 ± 0,000	10
	28	0,000 ± 0,000	10
	29	0,000 ± 0,000	10
	30	0,000 ± 0,000	8
	31	0,000 ± 0,000	9
	32	0,000 ± 0,000	6
	33	0,000 ± 0,000	6
	34	0,000 ± 0,000	9
	35	0,125 ± 0,125	8
	36	0,000 ± 0,000	9

[a] GC: Alive callus tissue; MF: Meristemoid formation; NoAB: Number of adventitious buds; ASD; Adventitious shoot development