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Clonal Propagation of the Desert Shrub, Haloxylon, for Pasture Improvement in Central Asia

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EXECUTIVE SUMMARY

Rangeland-based livestock production is the main form of agricultural production across much of semi-arid Turkmenistan. Desert rangeland carrying capacity varies significantly from year to year and season to season, sometimes taking a high toll on flocks and productivity. Perennial shrubs are the most stable rangeland fodder source and species of *Haloxylon*, endemic to Turkmenistan, are among the most valuable and productive fodder shrubs. Therefore, we set forth a number of research goals, the results of which could contribute to improving *Haloxylon* range.

Protocols were developed for prolonged storage of *Haloxylon* seed and for clonal propagation of juvenile plant material by micropropagation. Significant progress was made toward development of a system of propagation of *Haloxylon* by somatic embryogenesis. Studies were conducted of the phenology of the two target species, H. *aphyllum* and *H. persicum*, and methods for their cultivation and controlled use.

Six members of the faculty of the collaborating Turkmen institution visited the BIDR, most for extended periods of time. In addition to participating in the research, each also improved his/her command of the English language, gained an understanding of another culture and some attended various academic courses offered at the institute.

In addition to the training and experience of collaboration, the budget allowed the Turkmen institute to establish a tissue culture facility, for continued research.

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RESEARCH OBJECTIVES

Background

Desertification has increased in recent decades, threatening the future of almost a billion people. It is estimated that between 50,000 to 70,000km^2 of useful land goes out of production each year through desertification (GOK-DRSRS and UNEP, 1990; Le Houerou, 1981). ASALs of the former Soviet Union alone cover approximately 300 million ha., on which rangeland-based livestock production is the only viable form of agriculture. Indeed, the Central Asian arid belt comprises the vast majority of the former U.S.S.R.'s pastureland, supporting about 65 million head of sheep and goats (Babaev, 1986). Fodder produced on pastureland costs less than in any alternate system (Babaev, 1986; Babaev, et. al., 1986; Sanford, 1983), but unregulated grazing has contributed significantly to rapid degradation of arid lands (Balent, and Stafford Smith, 1992; Harrington, et. al., 1984; Krylova, 1986).

Turkmen desert range carrying capacity varies from year to year between 3.5 and 8.2 million sheep, depending on forage yields. Shrub pastures are the least prone to annual fluctuations in yield; grass ranges are the most prone (Payne et. al., 1987). Range improvement may be accomplished by planting selected perennial shrubs, converting ephemeroid range to year-round grazing lands and increasing carrying capacity significantly (Babaev, Manakov and Nikolaev, 1986).

Much work has been carried out in the deserts of Soviet Central Asia to identify promising fodder plants. Black and white saxaul (*Haloxylon aphyllum* and *H. persicum*), both of which occur in Central Asia, rank high among the species determined to be the most valuable fodder trees and shrubs, with high drought- and salinity-resistance (Nechaeva, et al. 1985). *H. persicum* usually grows on sandy ridge slopes and tops and can grow on a variety of soils, ranging from sandy to heavy clays. *H aphyllum* is larger than *H. persicum*, ranging from 4-7m. in height, as compared to *H. persicum's* 3-4m height, is more deeply rooted, more salt-tolerant and grows well in heavier soils than *H. persicum*. *H. persicum* is more drought resistant than *H. aphyllum* (Nechaeva, 1985). Sheep and cattle feed on the succulent young

shoots of both species in winter when their nutritional value is greatest. Camels feed on the shrubs year-round. The seeds are also an important source of nutrition. The wood is very hard and an excellent fuel, much valued in desert regions.

Both *Haloxylon* species are heterogeneous. Prior to the initiation of this project they could be propagated only from seed. Reports indicated that freshly harvested seed germinates well, but rapidly loses this capacity, with viability dropping to 10-20% after one year. This poses severe restriction on the timing of sowing, improvement, germplasm storage and establishment in new areas. Furthermore, many plants of little value for forage production occur among populations generated from seed. Superior individuals cannot be reproduced from seed; hence, development of methods of clonal propagation must accompany selection.

Overall and Specific Objectives

In view of the above, the overall objective set forth for this program was to contribute to the stabilization of animal husbandry by:

- selection of superior individuals of *Haloxylon aphyllum and H. persicum* in field trials in Turkmenistan and *in vitro*.
- studying phenology of the species in both countries, collection of climatic data from the regions of distribution and correlation with growth and development.
- development of a seed storage protocol that will preserve viability for extended periods.
- development of basic systems for mass propagation, including cuttings, micro-shoot proliferation and rooting, and somatic embryogenesis.

SEED STORAGE

Haloxylon shrubs enter the reproductive period at five to six years of age, producing fruit most abundantly at middle age. The seed is rounded, 2-2.5 mm. in diameter and coated with a thin transparent skin. It lacks endosperm, having a spiroid embryo with a pronounced yellowish rootlet and green cotyledons. Freshly harvested seeds generally have good germinating ability; however reports indicate that this rapidly declines during storage, to as little as 10%-20% after one year. It has been reported that good germinating ability may be extended by storage in a sealed vessel with potassium chloride. For this reason, the practice

has been to sow *Haloxylon* in late winter or early spring (February or early March), as soon as possible following maturation of the seed.

When we undertook this program there had been no reports of success in vegetative propagation of *Haloxylon*. Since propagation could only be from seed, and in view of the reported rapid loss in seed viability, we felt that the project should include development of a protocol for extending seed storage life. Such a procedure would allow for establishment of an *Haloxylon* gene bank as well as facilitate introduction of these valuable fodder species to other ASALs.

Methods and Results

Following preliminary studies on seed storage and germination conducted in 1993-4, seed of both species were collected in November 1994. *H. persicum* seed were collected from two shrubs on the campus of the Blaustein Institute. *H. aphyllum* seed were collected from several shrubs located about 100 km northeast of Ashkhabad, in the Karakum desert. Following the results of the preliminary studies, the seed of both species were stored at 4° C in the dark from as early as possible after the time of collection until use. The seed of *H. persicum* were placed under these conditions within minutes of harvest; those of *H. aphyllum* were stored at room temperature for the two-week period between collection and transport from Turkmenistan to Israel. Once in Israel, they too were stored at 4° C in the dark.

Further germination trials were conducted during 1997-98 with seed of *H. aphyllum* and *H. persicum* from the Karakul research station in Turkmenistan. The *H. aphyllum* seed had been harvested between 1994-1997. Those of *H. persicum* had been harvested in 1995. The seed of both species were stored in darkness at 4° C (in a refrigerator). Part of the *H. aphyllum* seed harvested in 1996 was stored in darkness at room temperature (~25° C). Germination rates were determined 5-7 days after imbibition.

The germination rate of fresh *H. aphyllum* seed harvested in 1997 was less than 10%; therefore it was not tested following storage. Seeds from the other harvest dates were germinated on 7 dates, two in June '97; two in July '97 and one each in February, April and

June '98. An additional germination test was done in January 2002 with *H. aphyllum* seeds from the 1994 harvest. A trial with seed of the 1996 *H. aphyllum* lot showed maintenance of good germination rates (and perhaps an increase in the rate) by refrigerated seed during two years, while seed stored at room temperature lost viability with increasing storage time, decreasing to nil at the end of two years (Figure 1). In germination trials with seed of *H. persicum* harvested in 1996 and of *H. aphyllum* harvested in 1994, 1995 and 1996, and stored at 4° C, high germination rates were maintained for up to 7 years (Figure 2).

Rates of seed germination varied among selections of *H. persicum* and between *H. persicum* and *H. aphyllum*. Viability was prolonged in all cases by cold storage. While fluctuation was observed in germination rates from date to date, on the whole rates remained between 83-95% after 3 and 4 years at low-temperature storage in darkness. Even after 8 years the germination rate of *H. aphyllum* seed was in the order of 90%. This is in contrast to earlier reports by others, which indicate that germination rate declines rapidly after harvest, even at reduced temperature storage. The results indicate, in contrast to previous information, that if handled properly, Haloxylon seed may be stored for extended periods and planted at convenient times in ASALs in other parts of the world.

PHENOLOGY OF *H. APHYLLUM* AND *H. PERSICUM*

biological and ecological

Haloxylon aphyllum is the main forest-forming plant of Central Asian deserts and considered to be the most valuable species for desert reclamation. It is a tree or a large shrub or tree, growing to a height of 4m with a crown width of 2-3m, and living generally from 50 to 70 years. *H. persicum* is a medium sized shrub, 3-5m high, with a somewhat shorter lifespan. Plants of both species are aphyllous and highly branched. Characterized by wide ecological amplitude, *H. aphyllum* occurs on a range of substrates, from well-drained sandy to heavy clay soils with deep ground water, to salinized soils with ground water near to the surface. It can grow under salinity as great as 2-3%; indeed salt, mainly sulfates, which at a concentration of 2% appear to stimulate growth of this species and contribute to its drought and heat resistance (Nechaeva, et al, 1978). While young shoots may die if exposed to temperatures in the range of -5 to 7°C, adult specimens withstand frost and temperatures of -

20 to -30° C (Kasyanov et al., 1982). The root system of *H. aphyllum* penetrates to depths of 14-16m., enabling efficient water use and stable nutrient supply under the harshest of conditions. *H. persicum* is more sensitive to fluctuations in water, salt, and aeration of the substrate. Its root system is less deep, generally penetrating to a depth of 4-5m, although roots have been found as deep as 20m.

H. aphyllum and *H. persicum* grow in separate specific niches, although in sandy deserts these may be in geographic proximity. *H. aphyllum* shrubs grow mostly in relief depressions, while *H. persicum* exhibits a propensity for sandy ridge slopes and tops, growing on stable to shifting, wind blown sands. It is less tolerant to low temperature than *H. aphyllum*, with the northern border of its distribution south of that of *H. aphyllum* (Nikitin, 1966).

Haloxylon species are distinguished for their extended growing period, which generally begins in February (sometimes as early as mid-January) and may continue through late November. Low air temperatures may result in a 3-4 week delay in commencement of the growing period. Shrubs coppice well after-felling, when dormant buds on remaining stumps produce numerous new shoots. Such shoots grow much faster than seedlings and enter the reproductive period earlier.

Efficient use of soil moisture and transpiration are important features of the physiology of *H. aphyllum*. During the hottest of months at Karakul (May through July) the average daily transpiration intensity of plants of different age ranges between 516-720 mg/hr/g of fresh weight, reaching 950 mg/hr at the time of highest air temperature and lowest relative humidity. These comparatively low transpiration rates fluctuate less over the season than those of other native species. *H. aphyllum*, like other shrubs belonging to the Chenopodiaceae, is also capable of positive balanced photosynthetic activity under temperatures as high as 38-42° C and low relative humidity, enabling it to continue its vital production of biomass during summer, with only limited shedding of assimilative twigs.

Flowering occurs in early spring, the timing influenced by weather conditions. The earliest date of the onset of flowering was in late February in Eastern Karakum (Repetek); the most

delayed flowering was in mid-April. The duration of flowering varies from two weeks to one month. Fructification occurs over a period of 1-2 months beginning between mid-September to early October. Between flowering in spring and fructification in autumn, a slow process of generative sphere formation occurs. A significant increase in size of the fruiting body occurs in October, concomitant with the onset of lower air temperatures, followed by the period of actual fruit formation, which lasts 10-12 days.

Fruit of the two *Haloxylon* species look very much alike, and are also similar to that of shrubs belonging to the genera *Salsola* and *Aellenia*. All bear membranous wings; the seeds, deprived of endosperm, have spiroid embryos coated with just a very thin film or membrane. An adult plant yields 0.2-10kg of fruit. 50-200kg of fruit may be harvested from non-cultivated hectare of *Haloxylon* forest.

uses

H. aphyllum ranks among the most productive forage plants (Nechaeva, 1958; Shamsutdinov and Ibragimov, 1983; Shamsutdinov, 1975). The nutritive value of the forage varies considerably over the year, ranging from 28 feed units in spring to 46 feed units in autumn and 37 in winter per 100kg of air-dry feed mass. *H. persicum* is also very drought-resistant and an excellent fodder shrub, distinguished for medium transpiration intensity, relatively high moisture content in the shoots, and considerable cell sap concentration. Camels feed on *Haloxylon* the year round; sheep readily feed *Haloxylon* in autumn and winter. Domestic livestock feed on shoots of the current and previous year and on the fruits, which are especially palatable and nutritious. This forage contains 10-12% protein (up to 29% in fruit), 2.2-2.7% fat, 21.2-38.6% ash, up to 39.3% nitrogen-free extractive substances, and up to 14.9% cellulose (on a dry weight basis).

environmental impact

Haloxylon communities are thin forest without canopy closure under which there is a unique microclimate. Light, wind and temperatures are modified significantly under the canopy. By filtering solar radiation, the turbulent heat exchange under *Haloxylon* crowns is 6-12 times lower than in the inter-crown space (Aisenshtadt and Orlovsky, 1976). The upper surface of

the crown of H. aphyllum detains 40-83% of total radiation, whereas H. persicum intercepts 56-60% of total radiation (Dedkov and Gunin, 1979).

H. aphyllum and *H. persicum* influence soil, grassy cover and its productivity. The fall and decomposition of *Haloxylon* shoots results in soil alkalization, affecting the plant species composition and productivity under the crown. Vegetation is totally lacking in the range of several meters from the trunk. Further out, concentric micro-belts (20-30 cm and more) of salt-resistant plants (*Suaeda lipskyi*) occur, followed by *Londesia eriantha*, *Schismus arabicus*, and finally forbs (*Maaecolnia granoliflora*, *Eremopyrum bonapartis*, *Bromus tectorum* etc.) develop; within 0.2m from the edge of the bare pattern *Carex physodes* grows (Lobko and Safonova, 1974).

conclusions

H. aphyllum and H. persicum are highly productive forage plants as well as an important source of fodder, firewood and raw materials. They are widely used to control desertification and in desert agroforestry in Central Asia. They are extremely resistant to low air and soil moisture content and high temperature. Their wide crown and large biomass production, has significant influence on the light, temperature and wind regimes beneath the canopy. The abundant amount of biomass that falls to the ground affects soil properties. This complex of factors creates a more favorable environment for many other plants and animals.

FIELD STUDIES

Field studies were conducted in Turkmenistan at the Karakul, Cele' and Repetek research stations. Most were carried out at Karakul, under the supervision of Dr. Mohammet Durikov. Methods used in pasture reclamation in low-rainfall regions were studied and adapted for implementation with *Haloxylon* species on takyr and sandy soils.

The Karrykul Field Station is situated at the southern tip of Karakum, 90km northwest of Ashkhabad, in an extremely dry southern desert sub-zone characterized by a very warm summer and mild winter. Annual air temperature averages 15.6°C, varying from 0.4°C in January (dropping to -28°C) to 31.6°C in July and reaching highs of 48°C. During the spring,

air temperature rises rapidly and precipitation increases. The hot and dry summer season begins at the end of April. Annual precipitation amounts to 148mm, most of it occurring during the autumn-winter period and only 4% from June to August. The soil dries considerably during the summer and in the first half of autumn, moisture reserves declining to 0-2mm. High air temperatures and low humidity contribute to generation of dry hot winds. Toward mid-September rains begin, marking the onset of an autumn season, characterized by conditions favoring renewed plant growth.

The soils of sand ridge slopes receive much less moisture than dune valleys. In February-March, the average moisture reserve, measured over a five-year period in the soil of dune valley sands, was equal to 32mm in a 100 cm layer; while the moisture reserve on the slopes of a sand ridge measured 19-21cm.

The station is on a slightly sloping clayey plain, covered in places by eolian sands. The area under observation extends 80km from north to south, and 30km from east to west. A unique feature of the station area is the ridge-takyr complex of Central Karakum: a combination of 10-15m long and 10-25m high sand ridges, stretching from north to south, and of depressions between the ridges. The depressions are occupied by takyrs, takyr-like plains (17%), and hillock sands drifted on the takyrs (68%), varying from 0.5-2m in thickness. Sand ridges cover 5% of the area. The texture of the soils is sandy, mostly fine- and medium-grain sand fractions. Physical clay accounts for 9%-12%, silt particles for 4%-6%. The content of salts and nutrients in sandy soils is negligible: humus, 0.2%-0.4%; total nitrogen, 0.01-0.02; mobile phosphorous, 5-10 mg-kg of the soil.

Both takyr and takyr-like soils occur side by side with ancient Tedjen delta deposits. The thickness of a takyr profile is 20-25cm. Most takyrs are structured by bedded clays and heavy loam deposits. Salinity of takyrs is high. The crust is usually somewhat alkalinized, and from 10-15cm downward the horizons contain considerable amounts of easily soluble salts. A 1m-thick layer is typically composed of 1%-3% dry residue, 4%-7% carbonates, 1% gypsum, and 0.5 humus.

Environmental Indices of the Karrykul Station

Elevation above sea level, m	90
Precipitation, mm	148
winter-spring	123
summer-autumn	25
Number of rainy days	40-45
Average annual temperature, °C	15.6
Average July temperature, °C	31.6
Average January temperature, °C	0.4
Frostless period, days	220-230
Average annual wind velocity, m s	2.9
Depth of soil wetting in winter-spring, cm	60-100
Depth of water table, m	20-25
Salinity of groundwater, g l	14-30

Three common plant associations occur in the area, each associated with a particular set of soil conditions:

on deeper sands: *Calligonum rubens - Mausolea eriocarpa - Carex physodes*

on thin sand drifts: *Salsola arbuscula - Artemisia kemrudica - Carex physodes*

on takyr-like soils: *Salsola gemmascens - Artemisia kemrudica - Gamanthus gamocarpus*

-effects of removal of above-ground biomass on shrub productivity: An important parameter in selection of perennial shrubs for fodder plantations is capacity to regenerate after heavy grazing. Experiments were conducted to simulate heavy grazing by cutting branches and above-ground parts of 2, 3 and 12 year-old shrubs of *H. persicum* and *H. apiculum* at the Karrykul and Sely field stations. Shrubs were pruned back to 80cm above the soil surface.

Shrubs less than three years old were heavily damaged by grazing and only recovered slightly. 11year-old *H. persicum* shrubs, prior to pruning, had an average height of 230cm, including 35cm long annual shoots. The crown diameter was 160cm. New shoots began to grow during the spring vegetation period following pruning, and by July the shrubs were densely covered with approximately 480 annual green shoots; 1.5 times more than on non-pruned shrubs. The new shoots had attained a length of 50 cm, resulting in a wide and low regenerated shrub, with a height of 120cm and a crown diameter of 90cm. Fruit was not produced until the second year after pruning. The pre-pruning biomass of *H. persicum* weighed 0.4kg. By the end of the growing season following pruning regenerated growth in the region accessible for

browsing (up to 120cm) weighed 2kg. Thus, a 5-fold increase in shrub productivity was affected by pruning or simulated grazing. In the third year post-pruning the crown increased in diameter to 130cm. The number of 1-year-old branches increased to 550 with a length of 55cm, while control plants had only 350 40cm long shoots. Available forage reached 3kg while that of the control plant was only 0.4kg. Four years after pruning shrubs had a dense crown of 650 1-year-old shoots, 55cm in length, and bore fruit. The fodder yield, four years after pruning, was 5kg, 4kg, of which was accessible to sheep, exceeding by 13 times the accessible biomass of control plants (Table 1).

In parallel trials with *H. aphyllum*, shrub height averaged 260cm prior to pruning, the crown diameter was 230cm, and annual shoots were 30cm long. Shrubs were cut back, as with *H. persicum*, to a height of 80cm. By July pruned shrubs were covered with new shoots. At the end of the growing season (November) the new growth, 400 shoots per shrub, had reached an average length of 50cm., almost twice the pre-pruning length, resulting in a shrub height of 130cm and crown diameter was 120cm. The total forage potential of the shrub increased considerably, reaching 6.5kg. During the following 3 years a large dense crown formed on experimental shrubs. The biomass accessible to sheep (from ground level to 120 cm) increased from 1kg to 5kg. The number of one-year-old shoots (forage mass) increased to 1100, while there were only 350 branches on the control shrub. The total green forage potential increased considerably, to 9kg air-dried material, while on the control only 4.5kg of fodder was available. Available green biomass (up to 120 cm above ground) from pruned and control plants was 6.5 kg and 1 kg, respectively (Table 2).

Haloxylon growing under natural conditions looses part of the annual shoots. A considerable part of the 2-year-old branches also die off in years with little precipitation. It has been established that *Haloxylon* has dormant buds but these do not become active if the tree is dry and new shoots do not appear when the main stem becomes old.

Our results demonstrate for both *H. aphyllum* and *H. persicum* that after very strong pruning, equivalent to heavy grazing, new branches begin to grow from these dormant buds on mature shrubs. This rejuvenation effect results, not only in the production of new vegetative biomass,

but also in increased productivity. Furthermore, the data suggests that more shrubs may be planted per unit area when grazed (if water is not limiting), as a consequence of the smaller crown diameter caused by grazing.

seedling preparation and field trials

After removal of the wings of the seed coat, seeds of *H. aphyllum* and *H. persicum*, were placed into a solution of 70% alcohol and 3% hydrogen peroxide for 5-7 min and washed on a stirrer for 3 hours, with changes of water every 30 minutes. They were then germinated in soil in 5 liter polyethylene sacs in a greenhouse at the Blaustein Institute in mid-February 1997. 4-5 seeds were sown per sac at a depth of 1-1.5 cm on a substrate of sandy soil mixed with some clay in the center of the sac. Sacs were irrigated daily from the time of sowing until the seedlings broken through the soil, after which they were irrigated on a weekly basis. The first shoots of both species appeared 3-5 days after sowing, and many had appeared by 5-8. Side shoots appeared 30-40 days after sowing.

Three months later, the seedlings were planted in heavy loess soil in the field, on a 2 X 2m grid, in 30 cm diameter x 70 cm deep holes. The plot was irrigated 5 days prior to planting, at the time planting and twice more in subsequent weeks. All trees survived and produced new shoots. The *H. aphyllum* were more vigorous that the *H. persicum* (Table 3). These differences in performance were as anticipated, since *H. aphyllum* occurs naturally on heavy soils, while *H. persicum* performs best on sandy soils.

influence of irrigation regimes on growth and development

In a second field trial, 64 *H. aphyllum* seedlings, germinated in the greenhouse, were planted out at the beginning April 1999, as above, in an experimental field measuring 10 X 22 m. A pre-planting irrigation of 25 l plant was given, as well as one 8 l plant post-planting irrigation. One row of seedlings was drip irrigated with 12 l water per bush every 2 weeks, a second row - once a month, a third row - once in 2 months. Three rows were not irrigated. Seedling height at the time of planting ranged from 11 to 18 cm, with an average of 14 cm.

All seedlings survived, regardless of the irrigation regime. After one year seedlings that did not receive additional irrigation were shortest (13 cm) while those irrigated twice per month were highest (55 cm). Seedlings irrigated once per month or once in two months differed little, growing to an intermediate height (47 cm). Lalymenko et al. (1998) similarly found, in studies carried out at the Nebidag research station in Turkmenistan, that irrigation prior to sowing and an initial irrigation after planting of seedlings is sufficient for establishment, and that subsequent irrigation did not influence growth and development of *H. aphyllum*.

Results of three years of field experimentation at the Jacob Blaustein Institute have revealed that:

- non-irrigated trees are almost half the height of irrigated trees; greatest height was attained by irrigation twice a month (81 cm);
- trees irrigated once a month or once in two months attain the same height three years after planting (58 cm). We thus conclude that application of 50 liters of water annually (12 l per irrigation once in two months) results in the same amount of growth as 97 liters given in 7 monthly irrigations per year;
- trees under stress (without irrigation) begin vegetative growth 12-15 days earlier than irrigated trees;
- the weight of 1000 seeds from non-irrigated trees is 1.6 times higher that of an equal number of seeds from irrigated trees.

A few of the *H. aphyllum* trees flowered and produced seeds in the third year after planting. Flowering occurred in mid-March. Seeds collected in 2000 in an experimental field at the Blaustein Institute from three-year old trees had very low viability: 1-5%.

Seeds were also planted out in trials at the Nebitdag Agroforestry Amelioration Station and the Repetek Desert Sand Station of the DRI. At the DRI Seeds without wings were held in 0.5% KMnO for 30 min. and then were washed in running water and dried. Soils in containers were watered 2-3 days before sowing, in order to moisten the substrate and to remove dissolved salts. Seedlings, prepared in a nursery, were planted out after two years on sands in the Karrykul field station in February. At the time of planting they were 20-25cm high with roots 30-40cm long. The sandy substrate was prepared by making 25-30cm deep

furrows and covering their borders with a thin layer (1cm) of clay. Seedlings were planted on the sandy bottom of a furrow. By autumn the seedlings had reached heights of 50-65cm.

On average, 1000 cleaned seeds of *Haloxylon aphyllum* without petals weighed 2.9gr. and 1000 cleaned seeds of *H. persicum* weighed 3.2gr. Seeds were sown 1-1.5cm deep; 4-5 per container, on a substrate of sandy soil mixed with some clay in the center of the containers. In this way, about 360 plants of *H. aphyllum* or *H. persicum* could be prepared per m^2. Containers were irrigated daily from the time of sowing until the seedlings broke through the soil, after which they were irrigated on a weekly basis. The first shoots of both species appeared 3-5 days after sowing, and many had appeared by days 5-8. Side shoots appeared 33-40 days after sowing. If seeds were sown in mid-January the seedlings were ready for planting out 65-70 days later.

afforestation techniques

Various techniques have been applied for afforestation of Central Asia pastures with low productivity. In some instances wide or narrow shelter-belts have been planted, in others massive land reclamation was undertaken in order to create long-term winter pasture. *Haloxylon* pasture-protection belts have contributed to productivity through the yields of *Haloxylon* itself, as well as by altering the microclimate, promoting expansion of ephemers and of summer saltworts within the belts and contributing to increasing yields of fodder on adjacent native ranges. On the whole, the cropping capacity of such pastures is 1.5-2 times greater than that of unprotected and unameliorated fodder areas (Nechaeva et al., 1985).

-afforestation in piedmont deserts: A system of pasture-protecting plantations composed of principal (longitudinal) and auxiliary (transverse) belts have been implemented with *Haloxylon*. The principal belts play a leading role in protecting desert pasture from wind, while the auxiliary belts serve to divide them into grazing areas. The longitudinal belts, which may be up to 25m wide, are laid down perpendicular to the prevailing winds on sandy loam and weakly loamy gray-brown soils. In locations where hydrological conditions (the water table at a depth of 5-35m) are favorable, the belt-to-belt interval is 250-300m. On medium-loamy and salinized light-textured soils the interval is 200-250m; on weakly and medium

salinized clay soils as well as on areas with deep groundwater, it is 100-150m (Nechaeva, Shamsutdinov and Ibragimov, 1985).

Ploughing is done to a depth of at least 20cm, in order to promote accumulation and retention of soil moisture, good aeration and availability of nutrients. The best time for ploughing is autumn-winter, after the soil has been moistened by rainfall. Sowing may follow between mid-December and mid-February (Shamsutdinov, 1975).

The optimal stand density for *Haloxylon* is 500-1000 trees ha. This rate has proven best for pasture protection as well as for encouraging development of ephemers and annual saltworts, providing for highest fodder productivity. Young plants grow very slowly, reaching a height of only 10-15cm, by the end of first season of vegetative growth. Since the rate of seedling survival to the end of first growing season is in the order of 50-60%, our experience has shown that seeding should be at the rate of 10-12kg ha.

-afforestation in takyr soil: Takyr lands in the arid zones of the world are concentrated largely on alluvial, proluvial, deluvial deposits and lands of ancient irrigation. They are usually characterized by flat relief, high salt content, very low water permeability and the presence of algae and lichens. The presence of higher plants is very limited as a consequence of the dryness of these soils due to their low permeability.

Technology for cultivating plants on takyr soils, by "harvesting" runoff water, was developed at the Nebitdag station, following traditional practices of the local population (Lalymenko et al., 1983). A system of large furrows is cut perpendicular to a main slope, directing surface runoff to the plants and creating moisture reserves in the soil. The shape of the cross-section and configuration of the furrows, the spacing between furrows and provision of a watershed area for each, and the morphology of the vertical profile of the takyr soil are critical to successful implementation of the system. Trenches with a triangular cross-section (an internal angle 95-110^0) and with double-breasted summits created by trenchers proved to be the most successful. Some time after establishment of the initial trenches, an additional furrow of equal size is cut near each of them, further enhancing the capacity for accumulating water in the

soil. The interval between furrows is 13-15m. Seeds are sown on the border of the furrow so that they are not submerged. "Granulated seeds" are sown on moving sands ("barkhan"). These are prepared by stirring the seeds in a 1:1 clay-sand slurry, before spreading on the sandy surface.

By the end of the fourth year of cultivation in twin furrows on takyr soils, 1.5-1.8 t ha. raw wood and up to 1.2 t ha of palatable green mass are produced by *H. aphyllum*.

SALINITY EFFECTS

Experiments were conducted at the Blaustein Institute to determine the influence of substrate salinity on growth and development of *H. aphyllum*. 7 days after germination, seedlings were planted in a greenhouse in 4 12 liter hydroponic vessels containing Hoagland's solution: 12 plants of per vessel. An additional 12 seedlings were planted in soil in plastic pots, to serve as a control treatment. At the end of one week, the medium was replaced in each hydroponics vessel. One treatment received standard Hoagland's solution; the other three were filled with Hoagland's plus 1%, 2%, and 4% NaCl. The solutions were changed 4 additional times at weekly intervals.

Plants died after 5 days in medium containing 4% NaCl. The other treatments were evaluated at the end of 40 days. The average height of the control plants at the end of 40 days was 8.9 cm (ranging from 5.3 to 12.4 cm); plants in 1% NaCl solution had an average height of 13.6 cm (9.5 to 19.3 cm) and those in 2% NaCl had an average height of 10.5 cm (6.0. to 14.0 cm). Plants grown in Hoagland's solution without added NaCl were more highly branched and slightly taller than those in the soil control. Those in 1 and 2% NaCl were more branched than the 0 NaCl treatment; the 1% NaCl was highly branched and significantly taller than all other treatments (Figure 3). The root system of the 1% NaCl treatment was longer than that of any of the other treatments, while at 2% NaCl root development was inhibited.

In preliminary trials *in vitro*, *H. aphyllum* seedlings were observed to germinate more vigorous and grow more rapidly on saline (200mM NaCl) culture medium than on non-saline medium. Preliminary studies also suggested that heat stabile proteins accumulate in seedlings growing on the saline medium but not in non-saline medium (D. Pelah, E. Birnbaum, Y. Heimer, A. Najedat and N. Orlovsky). Reports in the literature correlate such proteins with stress tolerance.

CLONAL PROPAGATION

Due to their heterogeneity, only a small percentage of *Haloxylon* shrubs among populations generated from seed are superior forage producers. In order to advance pasture improvement in Turkmenistan with selected cultivars and to facilitate introduction of selections of *Haloxylon* species to other ASALs, a method of vegetative propagation is needed.

CUTTINGS

Vegetative propagation of *Haloxylon* by cuttings has been attempted, with little success. There has been progress in vegetative propagation and micropropagation of other members of the family *Chenopodiaceae*, which includes other important fodder species (e.g., *Atriplex* and *Kochia*) and edible plants (beet, chard and spinach) (Atanassov, 1986; Detrez, et. al., 1989; Doley and Saunders, 1989).

Methods and Results

Shoots were cut from three and four month old *H. aphyllum* seedlings growing in a greenhouse and from two-year-old shrubs growing on the Sede-Boqer Campus of BIDR during the months of May-July. These were divided into 6-7 cm long pieces and kept in water. The basal ends were dipped in an aqueous solution of 1000ppm IBA or treated with 0, 500, 1000, or 2000ppm IAA for 5 min, 60 min, 6 hr, 12 hr, 24 hr or 48 hr. The controls were treated with water only. Cuttings were then inserted into plastic pots containing (a) peat moss, (b) sand and clay, 1:1 or (c) sand. They were kept in a greenhouse and watered on alternate days. Cuttings in sand were also held in the laboratory, covered with beakers to maintain moisture and watered on alternate days. Ten shoots were used in each treatment.

Cuttings wilted and started to die 9 days after having been transferred to pots. The upper portions of cuttings treated with IAA remained green and appeared healthy, but signs of necrosis appeared at the bases on the 9th day and by day 12 the cuttings were dead. None of the treatments induced rooting. The total failure is likely due to poor control of environmental conditions, and not necessarily total recalcitrance of the tissue. Shoots of a related species, *H. salicornicum*, were rooted without any phytohormone treatment (Kaul et al., 1992). This work should be pursued under well-controlled environmental conditions (humidity, temperature and light intensity).

TISSUE CULTURE

Tissue culture has become an important technique to reproduce crops that are difficult to propagate by seed and/or by conventional vegetative means. *In vitro* techniques offer several advantages over conventional practices of asexual propagation. Only a small amount of tissue is needed in order to regenerate millions of plants; many species that are resistant to propagation by conventional methods may be propagated *in vitro*; and *in vitro* stocks can be quickly proliferated regardless of season. *In vitro* techniques facilitate rapid multiplication of valuable genotypes, production of disease-free plants, germplasm conservation and easy international exchange (Govil and Gupta, 1997).

Micropropagation provides a practical method for cloning forest trees for afforestation. Significant progress has been made in micropropagation of the top 5 tree species, Eucalyptus, Acacia, Rubberwood, Radiata Pine and Teak (Gorst and Teasdale, 1999). We believe it also offers a practical solution for clonal propagation of selected fodder shrubs for rangeland improvement.

Vegetative propagation of woody plants by tissue culture may be accomplished by (a) enhanced axillary bud proliferation, (b) production of adventitious buds and (c) somatic embryogenesis (Murashige, 1974; Vasil & Vasil, 1980). Shoots are produced by the former two approaches and then are rooted. Somatic embryogenesis (addressed in the next section) leads to formation of an asexual embryo. Micropropagation via shoot multiplication is today

the better-developed procedure. Somatic embryogenesis, although generally more difficult to develop than a shoot multiplication system, seems to hold greatest promise for the long-term.

micropropagation

Members of the Chenopodiaceae that have been propagated through shoot proliferation from shoot tip and axillary bud cultures include *Beta vulgaris* (Hussey and Hepher, 1978), *Atriplex canescens* (Wochok and Sluis, 1980; Mei et al. 1997), *Chenopodium quinoa* (Burnouf-Radosevich and Paupardin, 1985) and spinach (Culafic, 1973). The process involves stages of establishment of culture, shoot multiplication, rooting and hardening off. The last two stages are sometimes combined and sometimes carried out *ex vitro*. The best approach and conditions for each stage must be determined for each species (Nehra and Kartha, 1994), however, the same patterns and principles generally apply (De Fossard, 1974, Thorpe, 1994).

BRP medium proved to be the best of several media tested for micropropagation of *Chenopodium quinoa*, (Burnouf-Radosevich and Paupardin, 1985). The medium of Hussey (Hussey and Hepher, 1978a) has been used to propagate *Beta vulgaris*. Linsmaier and Skoog medium (1965) has been used for *Atriplex canescens* (Wochok and Sluis, 1980b).

Preliminary efforts to initiate tissue cultures from shoots of *H. persicum* were unsuccessful. Efforts, therefore, were focused on micropropagation from seedlings germinated *in vitro*, with the intention of later implementing methods and media developed for seedlings on explants of adult shrubs.

Methods and Results

Experiments were conducted to establish cultures of *H. aphyllum* and to investigate the effects of media, phytohormones, and incubation conditions on various explant sources. Two basal media were tested, Murashige and Skoog macro- and micronutrients (MS, Murashige and Skoog, 1962) at full- and half-strength, supplemented with 1mg/l thiamine, 1mg/l pyridoxyine, 1mg/l Ca-pantothenate, 1mg/l nicotinic acid, 0.01mg/l biotin, 2mg/l glycine and 100mg/l myo-inositol (MS+vitamins) and BRP medium (which includes 10mg/l thiamine HCl, 1mg/l pyrridoxyine HCl, 1mg/l nicotinic acid, 4mg/l glycine and 100mg/l myo-inositol

[Burnouf-Radosevich and Paupardin, 1985]). All media contained 3% sucrose and were gelled with 2.4g/l phytagel (Sigma). Cytokinins and auxins were added individually and in combination in the various experiments. The pH of the medium was adjusted to 5.8 before autoclaving except in trials to determine pH effects. Ten ml aliquots of medium were dispensed into 25x100 mm borosilicate test tubes with plastic caputs and autoclaved at 121°C for 18 min. Cultures were incubated under a 16 hr photoperiod (50µmol m^{-2} s^{-1} cool white fluorescent light) at 24 ± 2° C, except in trials to determine effects of illumination.

Explants were derived from seedlings germinated *in vitro*, from 3-4 month old greenhouse-grown seedlings (collected in the months of May and June) and from a two-year-old shrub growing in the field (collected in March-May). The explants were approximately 1cm in length, composed of the cotyledonary node and adjacent stem tissue or axillary shoot sections with one or two nodes.

The seedlings were produced from seed of *H. aphyllum* from the Karakul field. Prior to disinfestation, the short wings of the seed coat were removed. Seeds were agitated for 5min in tap water containing a few drops of liquid detergent, rinsed for 2min in running tap water, again stirred for 5min in distilled water containing Tween 20 and rinsed five times in sterile distilled water. Seeds were disinfested with 1.58% NaOCl (30% commercial bleach) for 10, 20 or 25 min or in 0.05% (w/v) $HgCl_2$ for 1, 1.5, or 2 min, and then washed with sterile distilled water six times in aseptic conditions. After sterilization, seeds were inoculated onto hormone-free full-strength MS medium.

Stem sections from greenhouse-grown seedlings and from a shrub growing in the field were agitated for 10 min in tap water containing a few drops of liquid detergent. They were then rinsed in running tap water, stirred in sterile distilled water containing 0.1% benlate for 15 min and rinsed six times in sterile distilled water. The material was then disinfested with 1.58% NaOCl for 15, 20 and 30 min or with 0.05% (w/v) $HgCl_2$ for 3, 4 and 5min, or 0.1% $HgCl_2$ for 3.5, 4 and 5 min, and then washed with sterile distilled water six times in aseptic conditions.

Observations were made weekly and each experiment was terminated after 30 days. Data were recorded of explants producing shoots, number of shoots per explant, shoot length, number of shoots rooted and root length. The results are expressed as mean ± standard error. Mean values were calculated from ten replicates per treatment. Statistics were calculated using Statistix Version 2.0. The data were analyzed using analysis of variance (ANOVA). In each column means followed by the same superscripts are not significantly different. Means were compared by LSD at the 5% rejection level.

Disinfestation

-seed disinfestation No contamination appeared in the cultures when seeds were treated with 1.58% NaOCl for 25 min or with 0.05% $HgCl_2$ for 1.5 and 2 min. 20% contamination was observed when seeds were treated with 1.58% NaOCl for 10 min and 5% contamination was observed when seeds were treated with 1.58% NaOCl for 20 min or with 0.05% $HgCl_2$ for 1 min (Table 4).

96% of the seeds inoculated onto hormone-free MS medium germinated within 72 hours. The seedlings were of normal appearance, indicating that none of the disinfestations treatments caused damage to the embryo. Treatment with 1.58% NaOCl for 25 min or with 0.05% $HgCl_2$ for 1.5-2 min are considered generally appropriate for seed of *Haloxylon*. Inasmuch as $HgCl^2$ is highly toxic, the use of NaOCl is recommended whenever possible.

-axillary shoot disinfestation: Explants from the greenhouse and field remained green when treated with 1.58% NaOCl for 15 or 20 min, with 0.05% $HgCl_2$ for 3-5 min or with 0.1% $HgCl_2$ for 3.5-4 min, but browned when treated with 1.58% NaOCl for 30 min or with 0.1% $HgCl_2$ for 5 min. 80-100% of the explants from the greenhouse and field became contaminated within one week of being placed in culture when any of these disinfestation protocols was applied (Table 5).

Neither of the compounds, at the concentrations and durations of exposure tested, was effective in disinfestation of axillary shoots from plants growing in a greenhouse or the field. The research was continued, therefore, with explants (cotyledonary nodes and axillary shoots)

from seedlings germinated *in vitro*. Clones were produced from these in earlier stages of the work. Multiplication was through micro-shoot proliferation. Cultures ceased to produce elongating shoots as time passed, and, instead produced short multiple shoots. Short shoots differentiated from callus cultures derived from the above.

Shoot and bud induction on cotyledonary nodes and axillary shoots

Explants, approximately 1.0 cm in length, composed of cotyledonary nodes and adjacent stem tissue and 1-2 node axillary shoot sections from 40-day-old *in vitro* seedlings were used in all of the following trials, unless stated otherwise. These were cultured on full- or half-strength MS medium or on BRP medium supplemented with 0, 0.5, 1.0, 2.5, 5.0 or 10 mg/l BA, alone or in the presence of 0.1 mg/l IAA.

The analysis of variance of experimental data showed significant effects of growth regulators on various stages of shoot proliferation.

-*Shoot induction on cotyledonary nodes:* There was no shoot initiation from cotyledonary nodes in full-strength MS medium lacking IAA and BA and in half-strength MS containing 0.1 mg/l IAA but lacking BA. Shoot development did begin from cotyledonary nodes in all other treatments in the three basal media (Tables 6, 7, 8 and Fig. 4a), arising above the cotyledons at the cotyledonary node. Shoots that developed on full-strength MS + vitamins with any of the phytohormone regimes appeared to be greener and healthier in appearance than any which developed on half-strength MS and BRP media.

The greatest number of shoots from cotyledonary nodes occurred on full-strength MS + 2.5 mg/l BA, + - 0.1mg/l IAA (4.0 ± 0.33 [SE] and 4.6 ± 0.56 [SE] respectively (Fig. 4 and Table 6). Fewer were initiated on half-strength MS: the greatest number (3.3 ± 0.26 [SE]), as above, in the presence of 2.5mg/l BA without IAA (Table 7). On BRP medium an average of just 1.1 shoots was generated, the greatest number occurring in hormone-free media (Table 8). Shoot and bud length were greatest in half-strength MS media (Table 7). In all phytohormone-containing MS media, a hard compact callus developed at the base of the explant. Callus

development was greatest in MS with 5.0 and 10.0mg/l BA, and was significantly greater in media containing IAA. Callus development was distant from the point of shoot induction.

-*Shoot induction on axillary shoots*: The greatest number of shoots generated from axillary shoots was on full-strength MS supplemented with 1.0 mg/l BA with or without 0.1 mg/l IAA (2.2+/-0.41 [SE] and 2.0 +/- 0.36 respectively, Fig. 4b and Table 9). In half-strength MS, the greatest number of shoots was again in the presence of 1 mg/l BA without IAA (1.7+/-0.21 [SE], but there was no shoot production on hormone-free media (Table 10). In general, fewer shoots were produced from axillary shoots on BRP medium than on either full- or half-strength MS (Tables 9, 10 and 11). In all phytohormone-containing MS media, a hard compact callus developed at the base of the explant. Callus development was greatest in MS with 5 and 10mg/l BA, and was significantly greater in media containing IAA. Callus development was distant from the point of shoot induction.

-*Shoot and bud formation on MS medium +/- BA +/- IAA*: Shoot induction was found, above, to be most efficient on cotyledonary nodes cultured on full-strength MS medium with 2.5mg/l BA and on axillary shoots on 1.0mg/l BA with or without 0.1mg/l IAA. Cotyledonary nodes and axillary shoots were, therefore, cultured on full-strength MS medium supplemented with 2.5mg/l BA for cotyledonary nodes and 1mg/l BA for axillary shoots alone or in the presence of 0.001, 0.01 or 0.1mg/l IAA.

Little effect of low IAA concentrations (0.001, 0.01 and 0.1mg/l) was found on shoot and bud formation in the presence of BA (2.5mg/l), although callus development on both cotyledonary nodes and axillary shoots was greater in media containing IAA (Tables 12 and 13). The results, comparable to those in the previous experiment, demonstrate that exogenous IAA was not necessary for shoot induction from cotyledonary nodes and axillary shoots.

-*Shoot and bud induction on adventitious shoots generated in vitro*: In efforts to induce further multiplication, shoots produced *in vitro* from cotyledonary nodes on full-strength MS + 2.5mg/l BA and from seedling-derived axillary shoots on full-strength MS with 1mg/l BA, were subcultured on full- or half-strength MS and BRP media containing 0, 0.5 or 2.5mg/l

BA. At the end of four weeks of culture, the greatest number of shoots was produced from both types of explant on half-strength MS containing 0.5mg l BA (Fig. 5b and Fig. 6b). 11.0 ± 0.57 shoots developed per culture derived from cotyledonary shoots with additional length of 0.61 ± 0.01 cm per explant, while 5.6 ± 0.04 shoots developed per axillary shoot explant, attaining additional length of 0.46 ± 0.04 cm per explant. Shoots multiplied under these conditions from cotyledonary nodes developed in a compact rosette form (Fig. 4c). Higher concentrations of BA reduced the multiplication rate.

There was no multiplication or elongation from cotyledonary node explants on hormone-free full- or half-strength MS (Fig. 5a and b). Relatively few shoots developed from axillary shoot explants on hormone-free half-strength MS (2.0 ± 0.25 [SE], Fig. 6b) and there was no multiplication or elongation on hormone-free full-strength MS (Fig. 6a).

In BRP media with or without hormones, relatively few shoots multiplied on either explant type (Fig. 5c and Fig. 6c).

-effects of BA + - IAA: The greatest numbers of shoots were induced above on cotyledonary nodes in full-strength MS medium + 2.5mg l BA and on axillary shoots in full-strength MS medium + 1mg l BA, with or without 0.1mg l IAA. Here, the effects of IAA + BA on subcultured shoots were investigated. Shoots produced from cotyledonary nodes and from axillary shoots were transferred onto full-strength MS containing 0, 0.001, 0.01 and 0.1mg l IAA in combination with 1 or 2.5mg l BA. No multiplication or elongation was observed on shoots of either origin, and shoots became yellow 20 days after inoculation.

-pH effects: H. aphyllum has been reported to grow better in soils with a pH range of 6.0-8.1 (Rodin, 1977). In order to determine whether *in vitro* development might be improved at elevated pH, cotyledonary node and axillary shoot explants of *H. aphyllum* were cultured on liquid full-strength MS medium + 1.0mg l BA, with the pH adjusted to 5.8, 7, 8 or 9 before autoclaving. Also, shoots, produced from cotyledonary nodes and axillary shoots, were subcultured on half- strength MS liquid medium containing 0.5mg l BA the pH of which was adjusted to 5.8, 7, 8 or 9 before autoclaving. In both instances, neither multiplication nor

elongation was observed at any pH tested and shoots became yellow 20-30 days after inoculation.

Shoot Elongation

In efforts to promote shoot elongation, shoots produced from cotyledonary nodes and axillary shoots were cultured for an additional four weeks on hormone-free BRP, full-strength MS or half-strength MS + - 0.5mg/l BA. Half-strength MS with 0.5mg/l BA had promoted the greatest shoot elongation in the multiplication stage (Fig. 5b & 6b).

Elongation was greatest on hormone-free BRP medium (1.09 ± 0.05cm for shoots from cotyledonary nodes and 1.02 ± 0.05cm for axillary shoots), while the rates of multiplication were very low. No multiplication or elongation occurred in hormone-free full- or half-strength MS. Further elongation was not observed on half-strength MS with 0.5mg/l BA, although greatest shoot multiplication (7.2 ± 0.44 [SE] with shoots from cotyledonary nodes and 7.0 ± 0.49 with axillary shoots) occurred on this medium (Fig. 7).

-effects of GA_3: Gibberellic acid has been reported to induce shoot elongation in many species (Wochok and Sluis, 1980, 1980b). A range of GA_3 concentrations was tested in efforts to induce shoot elongation in cultures of *H. aphyllum*. Shoot clusters and individual shoots produced from cotyledonary nodes and axillary shoots, multiplied on half-strength MS + 0.5mg/l BA and from hormone-free half-strength MS, were cultured on BRP with 0, 0.001, 0.01 or 0.1mg/l GA_3 for four weeks and on BRP with 0.01 mg/l GA_3 for one, two or three weeks. At the end of the respective trial period, shoots were transferred to hormone-free BRP medium for an additional period. Shoots did not elongate on any of the treatments and became yellow 30 days after inoculation.

-effects of IAA: Since elongation had been observed in rooting trials, when axillary shoots of *in vitro* seedlings were cultured on BRP medium containing IAA, shoots produced from cotyledonary nodes and axillary shoots were cultured on BRP medium containing 0, 0.5, 1, 2.5, 5, 10 or 20mg/l IAA. No multiplication or elongation was observed and, as above, shoots became yellow 30 days after inoculation.

Shoot elongation is not always a necessary stage of a micropropagation protocol, but since shoots of *H. aphyllum* generated *in vitro* tend to be short, induction of extension was thought desirable. None of the efforts to promote elongation succeeded.

Rooting

Axillary shoots from 5-6 cm long *in vitro*-raised seedlings, 1-2 cm long with 1-2 nodes, were used for rooting, except where stated otherwise. Rooting experiments were carried out in light and in darkness (covered with aluminum foil) on media containing combinations of 2,4-D and BA.

-half-strength MS medium + 2,4-D + BA: Axillary shoots were first cultured on half-strength MS containing 0 or 2.0mg/l 2,4-D + 1mg/l BA and 5mg/l 2,4-D + 2.5mg/l BA for 7, 14, 21 and 28 days. At the end of the culture period they were transferred to hormone-free half-strength MS medium. Shoots started yellowing after 20 days in hormone-containing media in all of the conditions tested. No root formation was observed. Callus development was greater in media containing 5mg/l 2,4-D + 2.5 mg/l BA in the light for 28 days than in the dark. More callus was produced when shoots were transferred to hormone-free medium and shoots turned yellow after 15 days in hormone-free medium in all treatments tested.

-BRP medium + 2,4-D: Axillary shoots were also cultured on BRP containing 0, 0.5, 1, 2.5, 5 or 10 mg/l 2,4-D for 10 or 15 days. 50% of the shoots remained green and produced very small amounts of callus at the base. They were then transferred to hormone-free BRP. After 30 days the shoots kept in the dark turned yellow, while the shoots kept in light produced more callus. 30% rooting was observed in hormone-free medium following induction in 10mg/l 2,4-D for 15 days and 10% rooting in 5mg/l 2,4-D for 15 days in light. An average of two roots per shoot were produced. Roots reached a length of 0.5cm, were very brittle and broke off easily.

-half-strength MS or BRP media + IAA, NAA or IBA: Axillary shoots produced from *in vitro*-raised seedlings and shoots produced from the cotyledonary nodes and axillary shoots were

cultured on half-strength MS and BRP – 0, 0.5, 1, 2.5, 5, 10, 15 or 20mg l IAA, NAA or IBA for 7, 10, 15 or 20 days, and then transferred to the respective hormone-free medium.

Shoots kept in darkness turned yellow and died after 15 days without swelling or rooting. Rooting occurred only on BRP medium. During 7, 10, 15 or 20 days preincubation in a 16 hr light 8 hr darkness regime in BRP medium containing 0.5-20mg l IAA, swelling began at the bases of shoots. Shoot tips in BRP medium containing IAA for 15 and 20 days began to turn yellow after 15 days in hormone-free BRP medium. Shoot abscission was observed in high concentrations (15 and 20mg l) of IAA. Rooting generally occurred on hormone-free BRP medium, within 15 days after culturing. Only at the lowest IAA concentration tested (0.5mg l IAA) did rooting occur without subtransfer. A maximum of 50% rooting was observed in hormone-free medium following induction in 5 or 10mg l IAA for 10 days or in 0.5 and 5mg l IAA for 15 days (Table 14). 40% rooting was observed in hormone-free medium following induction in 2.5mg/l IAA for 10 days. 30% rooting was observed in hormone-free medium following induction in 20mg l IAA for 7 days or in 1, 2.5 or 10mg l IAA for 15 days (Table 14). In general 2-3 roots formed per shoot. They were very brittle and broke off easily (Fig. 4e). No rooting or callus formation was observed in half-strength MS and BRP media containing NAA or IBA or half-strength MS containing IAA. Shoot tips kept in half-strength MS and BRP medium containing NAA or IBA or half-strength MS containing IAA for 15 or 20 days began to turn yellow after 15 days. Shoots kept for 7 or 10 days in hormone-containing media began to turn yellow 15 days after transfer to hormone-free half-strength MS or BRP medium.

subcultured shoots from cotyledonary nodes and axillary shoots: were cultured on half-strength MS and BRP media containing 0, 0.5, 1, 2.5, 5, 10, 15 or 20mg l IAA, NAA or IBA for 7, 10, 15 or 20 days, and then were transferred to the respective hormone-free medium. Shoots kept in darkness turned yellow and died after 15 days without swelling or rooting. Rooting occurred only on BRP medium. Shoots kept in hormone-containing media began to turn yellow 7 days after transfer to hormone-free half-strength MS or BRP medium. A maximum of 30% rooting was observed on subcultured shoots in the presence of BRP – 5

mg l IAA after 15 days. The roots were very small (0.2 cm) and abnormal (Fig. 4f). No root formation was observed on media containing NAA or IBA.

-BRP medium containing IAA + NaCl: Due to the halophytic nature of this species, NaCl was tested in root-induction medium. Axillary shoots were cultured on BRP containing 5 mg l IAA + 1% NaCl for 7, 10, 15 or 20 days and then transferred to BRP hormone-free medium. BRP + 5 mg l IAA without NaCl was used as a control. Swelling was observed in shoots kept in light as well as in darkness after 10 days in IAA-containing medium. A maximum of 40% rooting was observed in hormone-free BRP medium following induction in 5 mg l IAA+NaCl for 7 days, 20% for 10 days and 10% for 15 and 20 days. In general 2-3 roots formed per shoot. The roots were very small (0.3 cm) and weak. No rooting was observed in the shoots kept in dark. Shoot tips began to turn yellow after 15 days in hormone-free medium.

IBA and NAA did not induce rooting, while a low concentration of IAA (0.5mg l) induced 10% rooting. 50% rooting was observed in hormone-free medium following a 10-day induction period in 5 or 10mg l IAA or a 15-day induction period in 0.5 and 5mg l IAA. 30% rooting was observed in hormone-free medium following induction in 10mg l 2,4-D for 15 days or 10% in 5mg l 2,4-D for 15 days in light. The combination of a period of induction of rooting in the presence of an auxin, followed by root development in auxin-free medium has been applied to other species. For example, rooting was induced when shoots of *Eucalyptus grandis* x *E. urophylla* were cultivated on medium containing IBA for 5-15 days and then transferred to the same hormone-free medium (Barrueto-Cid et al., 1999). While *Chenopodium quinoa* rooted at low frequency on media supplemented with IAA, NAA or IBA, the percentage of root formation was high in medium without growth regulators (Burnouf-Radosevich and Paupardin, 1985).

Clones generated *in vitro* showed a wide range of characteristics. Rates of shoot proliferation, callus development and regeneration, for example, appear to have a genetic basis, varying among clones and therefore may be selected for. This might lead to a tool for selection of characteristics which might find expression *in vivo* as well.

somatic embryogenesis

Somatic embryogenesis is a pathway of differentiation that results in the formation of organized embryo-like structures. Under appropriate culture conditions, these organized structures pass through globular, heart-, and torpedo-shaped stages, with morphology similar to that of their zygotic counterparts (Michler and Lineberger, 1987), developing into whole plants. Species of many genera will undergo the stages of embryogenesis up to the torpedo stage, but not all have been induced to develop further into plantlets (Stafford and Warren, 1991).

Somatic embryogenesis can be induced from callus, cell suspensions and protoplasts or directly from cells of organized structures such as a stem or zygotic embryo. Explanted tissues are thought to contain cells that have the capacity for embryogenic development: pre-embryogenic determined cells (PEDCs) and cells which require re-determination through a period in culture, induced embryogenic determined cells (IEDCs) (Merkle et al., 1990). Somatic cells that are themselves embryonic, or not far removed from the embryonic state, are generally more easily induced to undergo somatic embryogenesis than differentiated vegetative cells. Komamine and his associates were able to establish a high frequency and synchronous somatic embryogenic system in carrot by selecting small, round and cytoplasm-rich cells, and treating with auxin, zeatin, mannitol and high O_2 (Nomura and Komamine, 1985).

Factors such as light regime, concentration of components of the medium, plant growth regulators and pH allow competent cells to demonstrate their intrinsic capacity for organized development, toward somatic embryogenesis. (Harry and Thorpe, 1994). Auxin, usually a high concentration of 2,4-D, induces somatic embryogenesis by effecting rapid cell division. Its removal permits globular-stage somatic embryos to continue to develop. The latter stages of embryogenesis are usually hormone-autonomous (i.e. produce sufficient hormones endogenously) (Sung et al., 1979, Stafford and Warren, 1991).

The present study describes the effects of culture media, growth regulators and environmental conditions on induction and differentiation of embryogenic callus of *H. aphyllum*.

Methods and Results

Experiments were carried out with seeds of *H. aphyllum* harvested in Turkmenistan in 1997 and 1998 and stored at 4 °C in the dark. The seeds were prepared and disinfested in NaOCl, as described previously. The embryos were then extracted and germinated on the surface of a medium containing MS salts (Murashige & Skoog, 1962) with 3% sucrose and 0.24% Phytagel™ (Sigma Chemical Co.) in borosilicate (25 x 100 mm) test tubes; one embryo per test tube. The pH of the medium was adjusted to 5.8 – 5.9 prior to autoclaving at 121 °C for 20 minutes. Growth regulators, when used, were added before pH adjustment. Unless otherwise indicated, the culture conditions were 24 ± 2°C under a 16/8 hour light/dark photoperiod with cool white fluorescent lights (50 µmol m^{-2} s^{-1}). Germination and growth occurred on this medium for three weeks, during which hypocotyls of *in vitro*-germinated seedlings attained lengths of 20-40mm. Details of each experiment are presented with results.

Morphological observations of all experiments were made weekly and data on callus induction, proliferation and differentiation were recorded. The trial on effects of combinations of auxins and cytokinins on callus induction and proliferation was designed as a factorial arranged in completely randomized blocks and the data were analyzed by analysis of variance. Specific mean comparisons among treatments were analyzed using the LSD test.

Callus induction and proliferation

-from zygotic embryos: Zygotic embryos were excised and inoculated onto 10ml of MS basal medium supplemented with 1, 2, 4, 8 or 16mg/l 2,4-D in combination with 0.5, 1, 2, 4 or 8mg/l BA, respectively. A growth regulator-free treatment was established as a control. Each excised embryo was considered to be a replicate; ten replicates were established per treatment. Cultures were maintained for 4 weeks at standard environmental conditions.

Callus developed in all replicates of the two treatments with the lowest concentrations of 2,4-D and BA; more in the treatment with the higher concentrations. The calli were light green in color and appeared on the surface as transparent, round cells when examined under the dissecting microscope. With combinations of the two phytohormones at higher concentrations less callus was induced and in fewer of the replicates (Table 15). No callus formed in the hormone-free treatment, although some swelling of tissue occurred.

-from seedling explants under light and dark conditions: Inasmuch as the previous experiment was carried out under a 16/8-hour light/dark photoperiod, this experiment was conducted in order to determine the effects of light and dark on callus induction. 8-10mm long hypocotyl segments and cotyledons were placed on the surface of half strength MS or BRP media containing 3% sucrose and 0.24% phytagel and supplemented with 2mg/l 2,4-D + 1mg/l BA, in test tubes (25 x 100 mm). For the light treatment, illumination and photoperiod were as described for germination. For the dark condition treatment, test tubes were covered with aluminum foil and kept in the growth room under the same temperature as the light treatment. Each explant (hypocotyl segment or cotyledon) was considered to be a replicate; there were fifteen replicates per treatment.

Callus formed in all replicates of all treatments, in both basal media and under light as well as dark conditions (Table 16). More was produced in BRP than in MS medium; the greatest amount on hypocotyl segments in BRP medium under illumination (Table 16). The surface of the callus was observed under the dissecting microscope to be covered with round, transparent cells. After 4 weeks of culture, calli obtained from induction treatments were transferred onto the same medium and cultured in the same environment for an additional four weeks. When transferred to MS medium without growth regulators, this callus showed little increase in volume and after 2 weeks of culture nearly 50% of it had turned brown. On the other hand, callus transferred to hormone-free BRP medium proliferated and gave rise to transparent spherical cells and to some elongated cells.

-from seedling explants over different time intervals: On the basis of the results of the prior experiments, it was decided to determine the effects of time on induction of callus from

seedlings and the proliferation response after induction. Hypocotyls and cotyledons of *in vitro*-germinated seedlings were excised and placed on the surface of BRP medium containing 3% sucrose and 0.24% phytagel and supplemented with 2mg l 2,4-D + 1mg l BA. Each explant (hypocotyl segment or cotyledon) was considered to be a replicate; ten replicates were established per treatment. Light and dark conditions were evaluated as described in the previous experiment. Callus development was evaluated after 5, 10, 15, 20 and 30 days of induction after which the calli and subtending explant tissue were transferred to hormone-free BRP medium for an additional four weeks. Development was evaluated after the initial phase of culture on hormone-containing medium and after the second growth phase on hormone-free medium.

Callus development was induced in at least some of the replicates of all treatments of this experiment. In those cultures exposed to the hormonal treatment for a shorter period of time (5 and 10 days) callus developed in only some of the replicates and in only small amounts (treatments 1, 2, 6, 7, 11, 12, 16 and 17, Table 17). No callus proliferation was observed when these calli were subcultured to BRP growth-regulator-free medium for 4 weeks (Table 17). In treatments cultured on hormone-containing medium for longer periods (15, 20 and 30 days) more callus was produced: the greatest amount in those treatments where the explant was hypocotyl tissue cultured in the presence of phytohormones for 20 to 30 days, irrespective of the light/dark regime. In these treatments, callus proliferation was observed after subculture to BRP growth-regulator-free medium for 4 weeks (phase II), but less relative amount of callus was produced in comparison to phase I (Table 17).

-Effects of combinations of 2,4-D and BA or Kin on hypocotyls: The results obtained in the former experiments led us to establish this experiment, the purpose of which was to determine the best combination of 2,4-D and BA or Kin for callus induction and proliferation. Hypocotyl segments were placed on the surface of BRP medium in 15 x 90 mm disposable polystyrene petri dishes (Miniplast Ein-Shemer, Israel). The medium was supplemented with 0.1, 0.5, 1, 2 or 5mg l 2,4-D + 0.1 or 1mg l BA or Kin. 3 to 4 hypocotyl segments were placed in each petri dish. Each explant was considered to be a replicate. 6 petri dishes were prepared per treatment.

Callus induction was observed during 4 weeks of culture in all treatments. The greatest amount of callus was generated in the presence of 1mg l 2,4-D + 0.1mg l BA (treatment 3, Table 18). The treatments containing 2mg l 2,4-D + 0.1 and 1mg l BA (treatments 4 and 9) also produced significant amounts of callus. At the end of the 4-week period, each set of calli was subcultured to the same medium and same culture conditions, as they had developed under. This pattern of callus proliferation continued following transfer. The highest increase of callus corresponded to treatment 3, followed by the treatment containing 1mg l 2,4-D + 0.1mg l Kin (treatment 13). Treatments 4 and 9, initially produced a great amount of callus, but when subcultured to the same medium and treatments less callus was produced (Table 18). Round transparent cells were observed under the dissecting microscope in all treatments. Elongated cells were frequently observed in treatment 3, as well as in treatments with 2mg l 2,4-D + 0.1mg/l BA, 2mg/l 2,4-D + 1mg l BA, 1mg l 2,4-D + 0.1mg l Kin, 2mg l 2,4-D + 0.1mg/l Kin and 2mg/l 2,4-D + 1mg l Kin (treatments 4, 9, 13, 14 and 19, respectively).

-Effects of combinations of auxins and cytokinins: This experiment was designed to test the effects of several other auxins combined with cytokinins on callus induction and proliferation and to compare their effects to those of 2,4-D. Earlier experiments indicated that 1mg l 2,4-D together with 0.1mg l BA was the optimum combination for callus induction and proliferation. Seeds were surface sterilized and germinated as in previous experiments. Two to three 8–10mm long segments were excised from each seedling and placed on the surface of BRP medium containing 3% sucrose and 0.24% phytagel in disposable polystyrene petri dishes (Miniplast Ein-Shemer, Israel). Media were supplemented with 0.1, 1 or 5mg l 2,4-D, NAA, IAA or IBA alone or in combination with 0.1mg l BA or Kin. A growth-regulator-free-treatment was established as a control.. The pH of the media was adjusted to 5.9. Temperature, illumination and photoperiod were as described for prior experiments. The explants from each hypocotyl were placed together in one petri dish and incubated for four weeks. Each explant was considered to be a replicate; twelve replicates were established per treatment. The callus obtained from each induction treatment was transferred once onto the same medium and cultured in the same environment for an additional four weeks.

Primary callus was observed on the cut surfaces in all treatments of hypocotyl segments after 7 days of culture. In 2,4-D-containing treatments, explants split at mid-length and callus also developed from subepidermal tissues onto the surface of the explant. The calli that developed when 2,4-D was present was light green, while with other auxins (NAA, IBA, and IAA), alone or combined with BA or Kin, calli were white to brownish, sometimes with reddish spots and were more compact. The calli produced in the presence of and transferred to NAA, IBA or IAA turned completely brown and died following subculture.

The callus that formed on the media containing 2,4-D and BA was friable. Stereomicroscopic observation showed localized proliferation of cell aggregates composed of round, translucent cells on the surface of the callus. Calli and the localized groups of glossy cell aggregates on their upper surfaces treated with 2,4-D + BA invariably gave rise to more glossy, translucent, globular and slightly elongated protuberances than did those treated with 2,4-D + Kin. Callus produced on 2,4-D + BA medium, following subculture to hormone-free BRP medium, proliferated two morphologically distinct cell types, spherical and elongated, both of which increased in size and number (Fig. 9a). Elongated cells seemed to be detached from the mother tissue (Fig. 9b).

2,4-D induced the greatest callus proliferation response at each concentration tested and in each combination, with or without a cytokinin. The treatments most effective in callus proliferation contained 0.1 or 1 mg/l 2,4-D combined with 0.1 mg/l BA or 1 mg/l 2,4-D without cytokinin. A very high auxin-cytokinin interaction was found in treatments in which 2,4-D was combined with BA. There was a significant difference between the treatment with 1mg/l 2,4-D + 0.1mg/l BA and other treatments tested (Fig. 10).

In the control treatment, growth-regulator-free, tissue swelling was observed but no callus was formed. In addition, calli produced on 5mg/l 2,4-D medium alone or combined with 0.1 mg/l BA produced adventitious roots after passage to hormone-free BRP medium.

Differentiation of embryogenic callus

The effects of cytokinins, ABA, GA_3, casein, maltose, sucrose, activated charcoal, light intensity and low temperature on differentiation of embryogenic callus were studied in the following trials:

-Effects of growth regulators: Cytokinins and abscisic acid (ABA) have been reported to enhance the formation of embryogenic callus (Kreuger et al., 1996; Kulshreshta and Coutts, 1997; Lenzner et al., 1995; Ogita et al., 1999; Saunders et al., 1999; Schuller et al., 1989). In order to determine the effects of cytokinins and ABA on differentiation of embryogenic callus, segments of calli generated in the earlier trial of the effects of illumination and darkness, 2-5 mm in diameter, were transferred to MS and BRP media containing 1 or 2 mg l each of BA, Kin and ABA in petri dishes. Six segments per petri dish and 6 petri dishes per treatment were established. Culture conditions and pH were as in previous experiments.

Although, callus increase was observed in all treatments in which BA and Kin were present, it was greater on BRP than on MS. As indicated previously, calli used in this experiment came from hormone-free BRP medium in which elongated cells were present. However, after treatment with BA or Kin, observations under a dissecting microscope showed that cells on the surface of the callus were small, round and transparent. After 4 weeks, no increase in the amount of callus or morphological changes were noted and the callus turned completely brown in cultures on both basal media containing ABA.

--casein, maltose and sucrose, + - growth regulators: Callus proliferation was obtained in the previous experiment in all treatments in which BA and Kin were added, but differentiation was not observed. There are reports that combinations of cytokinins with different carbohydrates and casein enhance differentiation of calli (Cabasson et al., 1995; Canhoto et al., 1999; Choi et al., 1998; Ignacimuthu et al., 1999). Induction of embryogenic tissue and embryo development by GA_3 and ABA has also been reported (Atree et al., 1998; Gupta and Timmis, 1998; Ogita et al., 1999; Saunders and Tsai, 1999). Therefore, the effects of Kin, BA, GA_3 and ABA on differentiation of embryogenic tissue in BRP medium were evaluated.

Calli from the trial on the effects of time on induction of callus from seedlings were used for this experiment. Each growth regulator, Kin, BA GA_3 and ABA, was tested at concentrations of 0.1, 0.2, 0.5, 1, 2 or 5 mg/l. Casein, at 50 or 500 mg/l was tested in combination with sucrose or maltose, with and without growth regulators. Maltose was tested in media in which sucrose was not included. Phytagel was used at the standard concentration of 0.24%. Six segments of callus ranging in size from 2 to 5 mm were placed on the surface of medium. Six petri dishes per treatment were established. As control, a hormone- casein- and maltose-free treatment was established. Culture conditions and pH were maintained as in prior experiments. Calli treated with casein were subcultured again in the same medium and exposed to the same treatments for a period of 4 weeks.

Callus proliferation was observed in all media in which BA and Kin were combined with casein, maltose or sucrose. About 40% of the calli in these treatments turned brownish at the end of the 4 week culture period. No callus increase was observed in the presence of ABA and GA_3 at any concentration and in combination with sucrose or maltose and casein. Calli turned brownish after 2 weeks of culture. 1mg/l Kin combined with casein at any concentration, and treatment with casein without growth regulators, showed morphological changes of tissue under the dissecting microscope: elongated cells gave rise to cylindrical structures with a glassy appearance.

Treatment with 500mg/l casein produced a very small increase in callus. The callus was whitish, and, under the microscope, showed sectors on the upper surface with round and elongated cells that had an opaque, rather than transparent, appearance. At the beginning of culture these structures on the upper surface were of glossy appearance; after casein treatment they turned whitish. After 2-3 weeks of culture, approximately 75% of the calli started to turn brownish and after 4 weeks culture 100% appeared brownish.

Maltose without growth regulators produced a whitish callus that under the microscope showed, on its upper surface, round and elongated white cells. Transparent cells were also observed. All calli turned brownish after 2 weeks of culture.

--casein + activated charcoal: The results obtained in previous experiments, as well as data reported on differentiation and minimization of browning of calli (Aberlenc-Bertossi et al., 1999; Blanc et al., 1999; Cabasson et al., 1995; Gleddie et al., 1982; Hristorofoglu et al., 1995; Verhagen and Wann, 1989) led us to test various levels of casein hydrolysate (50, 100, 250 and 500mg/l) in combination with 1g/l activated charcoal in media without growth regulators. Calli from the trial on the effects combinations of 2,4-D and BA or Kin on callus induction and proliferation from hypocotyls were used. The medium, containing 3% sucrose, was tested in semi-solid as well as liquid form. Semisolid medium was jelled with 0.24% Phytagel and distributed in polystyrene petri dishes (Miniplast Ein-Shemer, Israel). Six segments of callus, 2-5 mm in diameter, were placed on the surface of medium. Six petri dishes per treatment were established. Liquid medium was distributed in 250 ml Erlenmeyer flasks (Kimax, USA), (2 flasks per treatment). Approximately 1 cm^3 of callus was placed in 100 ml of liquid medium. Flasks were placed on an orbital shaker at 150 RPM. The same culture conditions as for previous experiments were maintained. Calli treated with 500mg/l casein + charcoal, were subcultured again in the same medium and grown for 4 weeks.

The increase in amount of callus was very small at all casein concentrations. Callus ranged from whitish on its upper surface in 250, 500mg/l casein to very light brown in 50, 100 mg/l casein. Round and elongated white cells were observed under the microscope. With 500mg/l casein and in the presence of activated charcoal, some small areas with a few green dots were observed. At the end of three weeks of culture, calli turned brownish in treatments without charcoal while those treatments in which charcoal was present turned light brown after 4 weeks of culture.

Segments of calli disintegrated readily after they were placed in liquid medium. Suspended individual cells and tiny clumps were observed in all treatments. As in solid medium, calli without activated charcoal oxidized and turned brown faster than those with charcoal. In the absence of charcoal calli looked brownish after 1 week of culture, while those with charcoal remained light green for 2 –3 weeks.

--*low temperature + low light intensity*: From results obtained in previous experiments and literature reviewed, it was decided to test the influences of light intensity and low temperature on embryogenic callus differentiation. Embryogenesis is very sensitive to light and temperature. Light is known to affect somatic embryogenesis through its effect on induction and on some morphological characteristics of differentiated somatic embryos (Arumugan and Bhojwani, 1989). Cold treatment (4° C) has contributed to major advances in the normalization of somatic embryo development and germination in asparagus (Kunitake et al., 1997). Calli masses produced on BRP medium supplemented with 0.1-5.0mg l 2,4-D, NAA, IAA or IBA + - 0.1mg l BA or Kin were transferred to BRP medium without growth regulators. After 4 weeks another subculture was made onto fresh medium of the same composition and cultures were grown for an additional 4 weeks. The calli produced were then transferred to BRP medium containing 500mg l casein + 1g l activated charcoal and kept at 4°C during three weeks under low light intensity (7 µmol m^{-2} s^{-1}) or in total darkness. Also, some calli were kept under illumination and temperature regimes as for previous experiments. Six petri dishes per treatment were established.

Incubation of calli for three weeks at 4° C in BRP medium supplemented with casein, under either low light intensity or total darkness, resulted in a general change in appearance. Under the dissecting microscope, areas of the callus on or near the surface appeared to become organized into white regions and round structures with deep green centers were observed (Fig. 9 C). Calli kept under standard culture conditions, illumination (50 µmol m^{-2} s^{-1}) and temperature (24± 2°C) produced a whitish callus that, as in the experiment to determine the effects of casein plus activated charcoal, started to become brownish after 3 weeks of culture. Round and elongated white cells with a few green dots were observed under the microscope.

Those calli exposed to cold treatment and low light intensity turned completely brown after being placed under the standard light intensity and temperature used for other experiments.

Discussion

Somatic embryogenesis has been accomplished with many plant species, including *Beta vulgaris* (Kulshreshtha and Coutts, 1997; Saunders and Tsai, 1999), another species of Chenopodiaceae. (Stafford and Warren, 1991; Perez J., 1998; Thorpe, 1994). In some species somatic embryogenesis occurred directly from the zygotic embryo, in others via an intermediary callus phase (Thorpe, 1994). The use of multiple hormonal sequences was reported to be necessary for induction of somatic embryogenesis in sugar beet callus and suspension cultures (Saunders and Tsai, 1999). Production and partial germination of somatic embryos from leaf-disc-derived callus of a fodder beet cultivar, on the other hand, was accomplished in hormone-free medium (Doley and Saunders, 1989). In Beta vulgaris, cotyledons from mature zygotic embryos were a good source of material for the induction of direct somatic embryogenesis (Kulshreshtha and Coutts, 1997).

Auxin and nitrogen play crucial roles in manipulating embryogenesis, modulating the switch between callus proliferation and embryogenesis in cell cultures (Halperin and Wetherell, 1964, as cited in Sung et al., 1979). In the presence of an exogenously supplied auxin, usually 2,4-D, callus growth is maintained, while when auxin is removed from the medium, embryogenic masses (or embryogenic clumps, proembryos or proembryonic tissue) develop into embryos (Thorpe, 1994, Ambazhagan and Ganapathi, 1999; Atree et al., 1991; Kim et al., 1999; Saunders and Tsai, 1999; Tautorus et al., 1991; Trick and Finer, 1999; Venkatachalam et al., 1999). In general, a combination of auxin with a lower level of cytokinin has been widely used for initiation of embryogenic callus. An active callus proliferation response was observed in *Eryngium foetidum* on a medium with 2,4-D combined with BAP, whereas when 2,4-D was used alone little callus formed (Ignacimuthu et al., 1998). A similar response was found in *Aesculus Glabra* Willd, since embryogenic callus was formed on media containing 2,4-D and Kin., while 2,4-D alone was not sufficient for induction of embryogenic tissue (Trick and Finer, 1999).

Our results demonstrated the effectiveness of certain auxin-cytokinin combinations in the induction and proliferation of callus in cultures of *Haloxylon aphyllum*. The treatment most effective in callus induction and proliferation contained 1mg l 2,4-D and 0.1 mg l BA,

Characteristics associated with embryogenic callus, glossy, globular structures and elongated suspensor-like cells, developed after transfer of the calli that had been induced on 2,4-D + BA medium to auxin-free BRP medium. The combination of 2,4-D and BA, thus, appears to have enhanced embryogenic potential of callus (Fig.9).

In *Solanum melongena* the cytokinins kinetin, BAP, 2iP and zeatin inhibited embryogenesis in leaf explant cultures, although they stimulated callus proliferation (Gleddie et al., 1982). Inhibition of embryogenesis by cytokinins was also observed in eggplant, petunia, Nigella, Citrus, Daucus, Panax and Perilla. Cytokinins have, however, been implicated as a necessary factor for somatic embryogenesis in some species (Gleddie et al., 1982). Kin and BA did not promote differentiation in *H. aphyllum* callus treated with the concentrations used in our experiment. Callus proliferation was observed but not morphological changes leading to embryogenic callus formation.

Two distinct types of somatic embryogenesis have been recognized, direct embryogenesis, in which a single cell commences meristematic growth and all the descendants of this cell form part of the embryo and indirect embryogenesis, in which an embryo develops from one or a group of cells in a previously formed meristematic cluster (Stafford and Warren, 1991). It appears that somatic embryogenesis in *H. aphyllum* is indirect, since initial cell aggregates gave rise to elongated cells from which small clumps were formed. This pattern resembled that described by Ogita et al. (1999), where embryogenic cell masses, PEMs (pro-embryogenic masses), were composed of small distinctive clusters of small, dense, cytoplasm-rich cells and elongated suspensor-like cells. These cell clusters showed great capacity for both proliferation and differentiation into somatic embryos. Therefore, if one can selectively isolate and culture such clusters from embryogenic tissues, it should be possible to elucidate morphological, physiological, and molecular changes in plant cells leading to embryo formation (Ogita et al., 1999).

Regarding nitrogen as a media component, it was reported in *Abies alba* that from 61 cell lines initiated, 36 have been propagated on proliferation medium with organic nitrogen (casein hydrolysate) for more than two years. Half of these cell lines showed increased

embryogenic potential, as indicated by the formation of globular embryos on the proliferation medium. Addition of 500mg/l of casein hydrolysate (CH) carried embryo development to the globular stage. 1000mg/l of CH proved to be significantly better than 500 mg/l, indicating that developing somatic embryos in later stages are highly dependent upon an external supply of organic nitrogen (Hristoforoglu et al., 1995). There are reports for other species as well, such as *Eucalyptus dunni*, of somatic embryo development being accomplished after embryogenic calli were subcultured in auxin-free media enriched with 1g/l CH (Termignoni et al., 1996). In oil palm and *Pinus nigra*, embryogenic development was achieved following the transfer of cell clusters onto plant growth regulator-free medium supplemented with 30g/l sucrose and 0.5g/l CH (Aberlenc-Bertossi et al., 1999; Radojevic et al., 1999). These findings support our results, in which casein hydrolysate played an important role in promoting morphological changes in embryogenic callus in a medium lacking growth regulators.

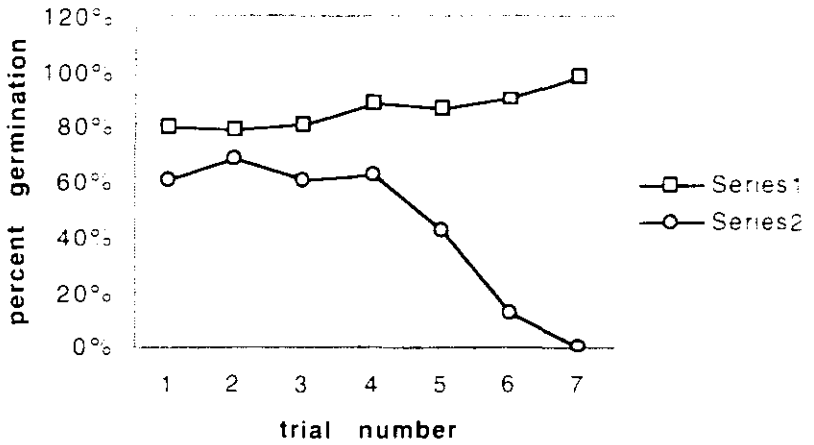
Light intensity and temperature effects on somatic embryogenesis have been demonstrated in studies with several plant species. In *Podophyllum hexadrum*, embryogenesis was completely suppressed if the temperature was raised above 27° C or the cultures were transferred to light, and resumed when cultures were returned to darkness (Arumugan and Bhojwani, 1989). Cold treatment (4° C) has contributed to major advances in the normalization of somatic embryo development and germination in asparagus (Kunitake et al., 1997). Embryo formation in tissue cultures of *Dysosma pleiantha* is highly sensitive to light. Calli kept in light for a month turned brown and lost the potential to differentiate embryos (Arumugan and Bhojwani, 1989). When calli masses were transferred to BRP medium containing casein under either low light intensity (7 µmol m^{-2} s^{-1}) or total darkness, at 4° C, they underwent a general change in appearance. Areas of callus on or near the surface seemed to become organized into white regions; and round structures with green meristem-like centers were observed. Light intensity did not seem to have an adverse effect on callus induction in *H. aphyllum*; the greatest amount of callus was achieved under illumination (50 µmol m^{-2} s^{-1}) and browning was not observed at this stage.

Callus of *H. aphyllum* showed two morphologically distinct cell types: spherical and elongated, both of which were capable of proliferation. Similar elongated cells, reported in

embryogenic cultures of other species, were characterized as suspensors or suspensor-like structures, which often were loosely attached to the mother tissue.

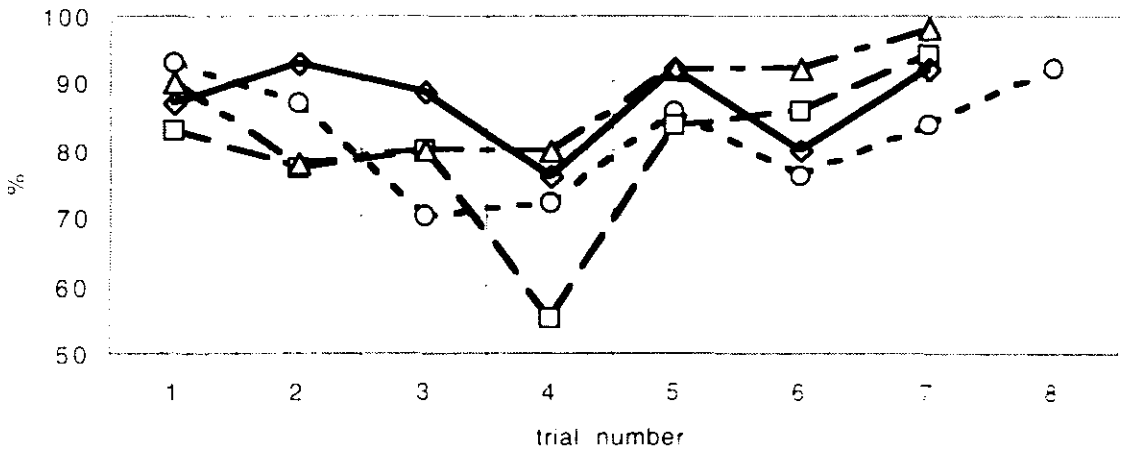
Embryogenic cultures have been characterized by the presence of whitish, compact and organized embryogenic sectors (Termignoni et al., 1996), or compact and white embryogenic bodies (Van Le et al., 1997), which were scored as somatic embryos. Kim et al. (1999) describe white, friable calli with nodular structures that gave rise to somatic embryos when transferred to an appropriate medium. White, friable callus developed green meristematic centers, which later became somatic embryos (Gleddie et al., 1982). These features occurred in cultures of *H. aphyllum* induced by the series of treatments described in this study; cell clusters, proliferated in auxin-free medium and, following cold treatment, differentiated into white bodies with deep green meristematic centers. On the basis of the similarity these culture bear to embryogenic calli reported in the literature, we believe this tissue holds the potential to produce somatic embryos. The results of the present study establish the basis for further research toward development of a system of somatic embryogenesis of *H. aphyllum*.

Figure 1. Germination rates of *H. aphyllum* seed harvested in 1996 and stored in darkness at 4°C (Series 1) or at room temperature (26° C, Series 2).



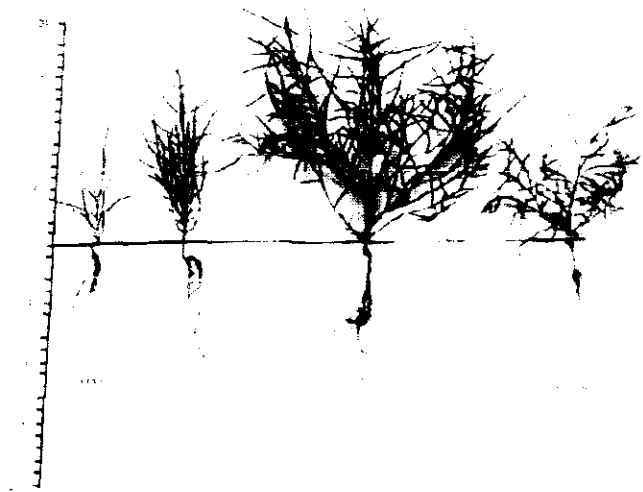
Seeds were germinated on 7 dates (trials 1-7): 1) June 4, 1997, 2) June 14, 1997, 3) July 18, 1997, 4) July 25, 1997, 5) February 8, 1998, 6) April 8, 1998 and7) June 9, 1998.

Figure 2. Germination rates of *H. aphyllum* and *H. persicum* seed harvested in 1994, '95 and '96, and stored at 4° C.



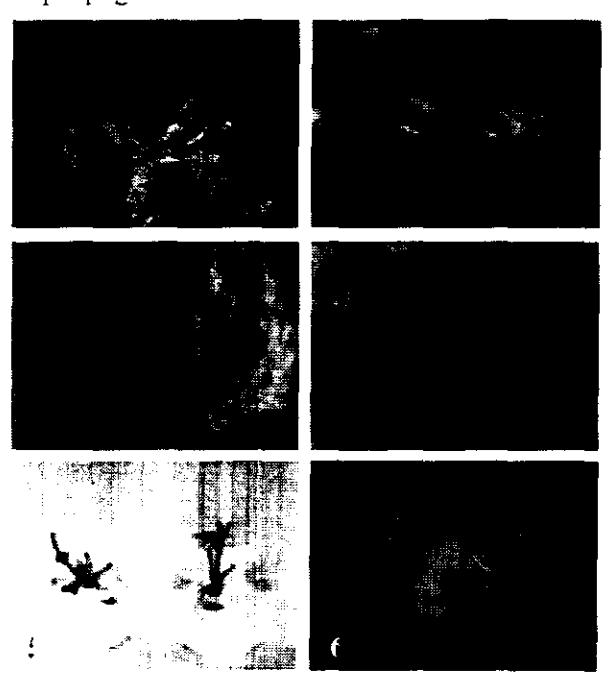
Germination trial				Legend	
#	date	#	date	symbol	seed lot
1	4.06.97	5	8.02.98	O	1994 *H. aphyllum* seed
2	14.06.97	6	8.04.98	—	1995 *H. aphyllum* seed
3	18.07.97	7	9.06.98	△	1996 *H. aphyllum* seed
4	25.07.97	8	1.01.02	⋮	1994 *H. persicum* seed

Figure 3. Haloxylon aphyllum seedlings grown for 40 days in Hoagland's solution + NaCl.



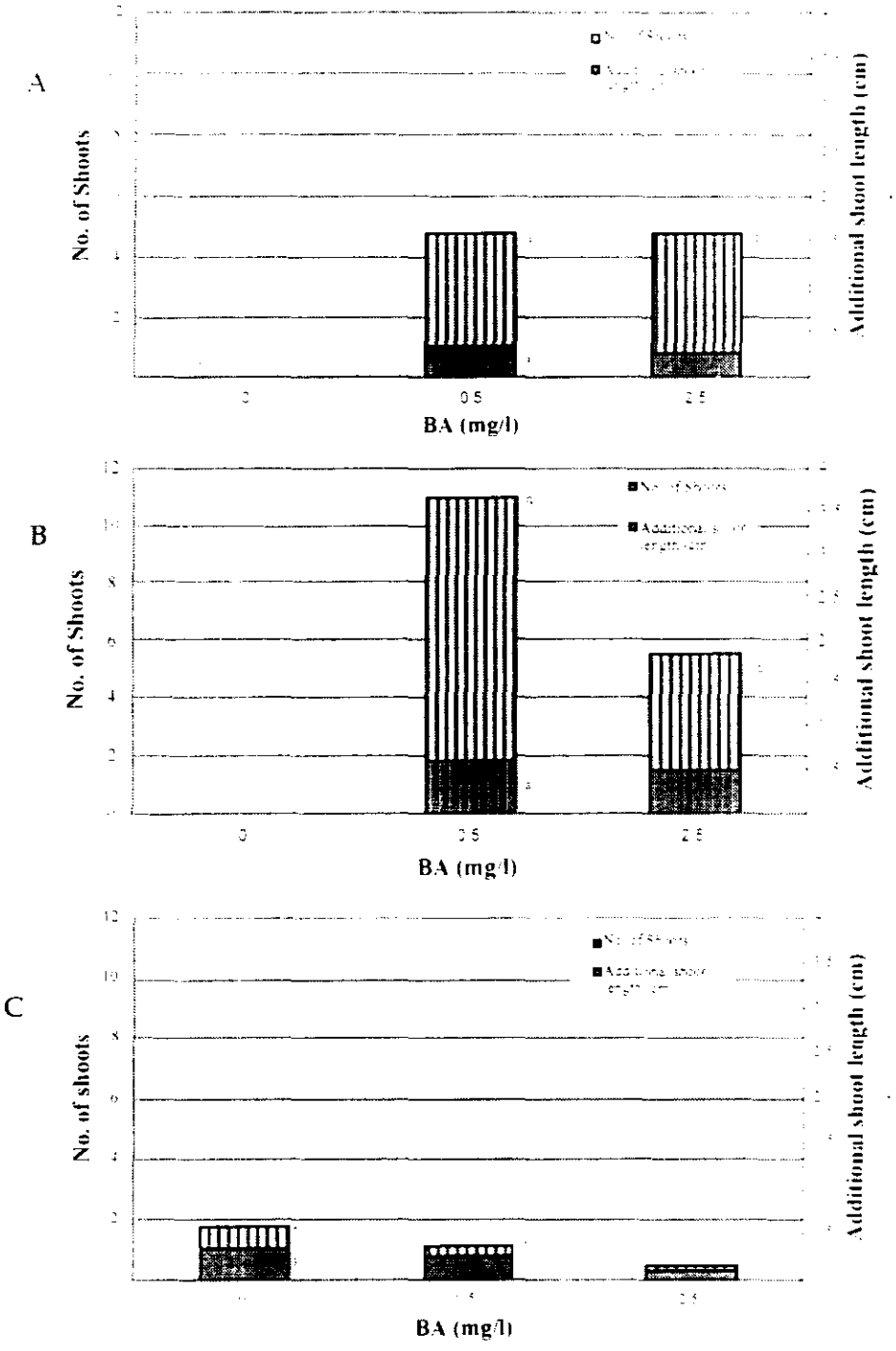
Control = soil; Hoaglands solution treatments, added NaCl: 0%, 1% and 2%

Figures 4. H. aphyllum propagation *in vitro*



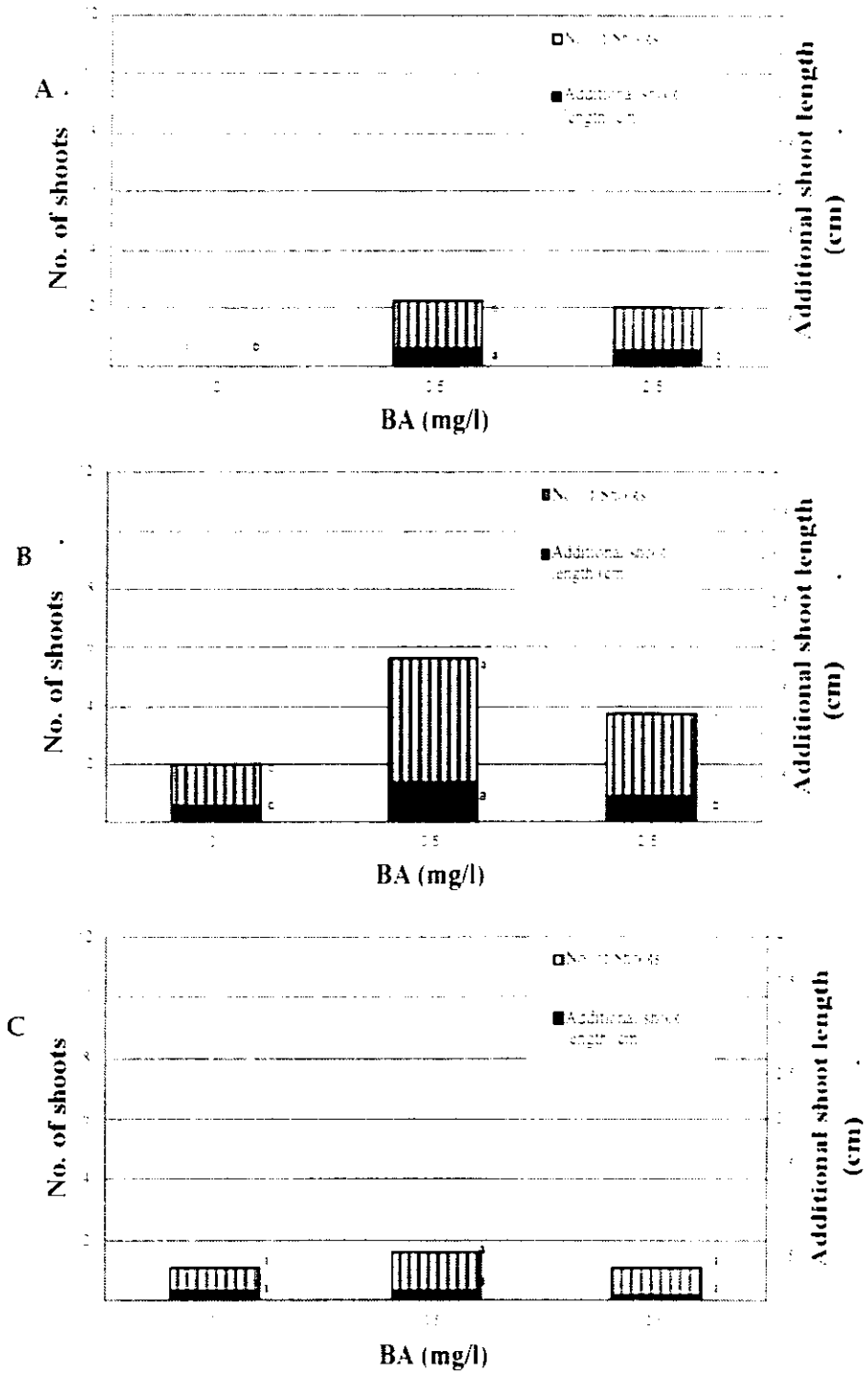
a. top left: shoot induction on a cotyledonary node on full-strength MS containing 2.5 mg l BA.
b. top right: shoot induction on an axillary shoot on full-strength MS containing 1.0 mg l BA.
c. center left: shoot multiplication on half-strength MS containing 0.5 mg l BA.
d. center right: shoot elongation on hormone-free BRP medium.
e. bottom left: rooting of axillary shoots derived from *in vitro* seedling on hormone-free BRP medium.
f. bottom right: rooting of a subcultured shoot on hormone-free BRP medium.

Fig. 5. Effects of BA on shoot proliferation from cotyledonary nodes of *H. aphyllum*



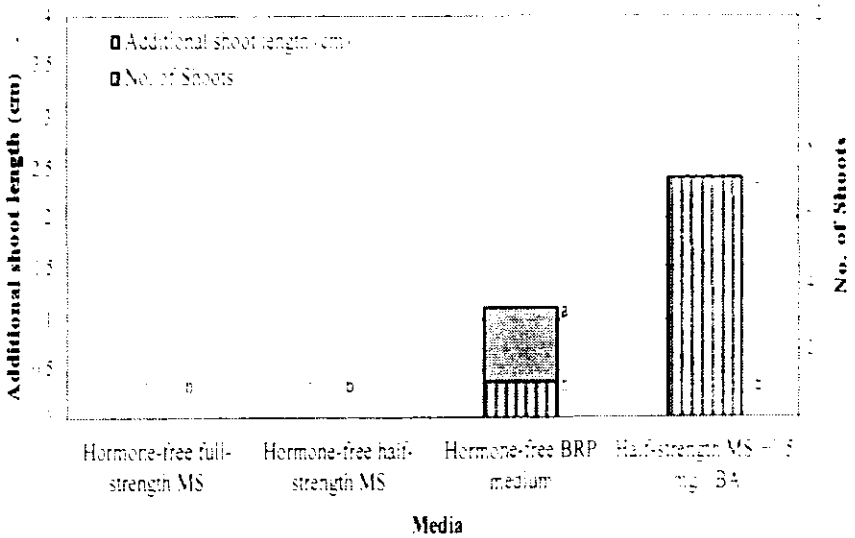
(A) on full-strength MS, (B) on half-strength MS and (C) on BRP medium.
Values represent mean ± SE. Columns with different letters are significantly different at $P = 0.05$ using LSD test.

Fig. 6. Effects of BA on shoot proliferation on axillary shoots of *H. [illegible]*



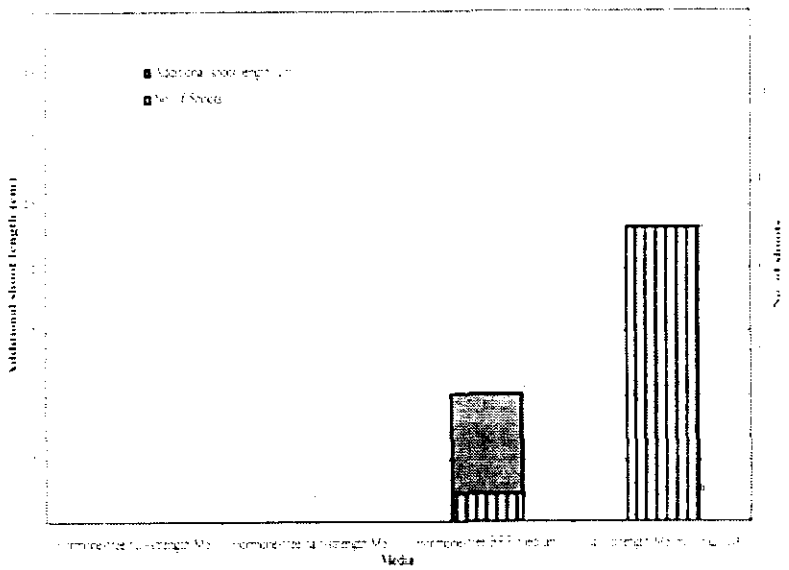
(A) on full-strength MS, (B) half-strength MS, and (C) BRP medium.
The values represent mean ± SE. Columns with different letters are significantly different at P 0.05 using LSD test.

Fig. 7. Effects of BA and subculture media on shoot elongation and new shoot proliferation from cotyledonary nodes of *H. aphyllum*.



Values represent mean ± SE. Columns with different letters are significantly different at P [illegible] 05 using LSD test.

Fig. 8. Effects of BA and medium on shoot elongation and new shoot formation from axillary shoots of *H. aphyllum*.



Values represent mean ± SE. Columns with different letters are significantly different at P 0.05 using LSD test.

Fig. 9 Embryogenic callus in *H. aphyllum*.
A. Spherical cells. B. Suspensor-like cells. C. Green meristematic centers.

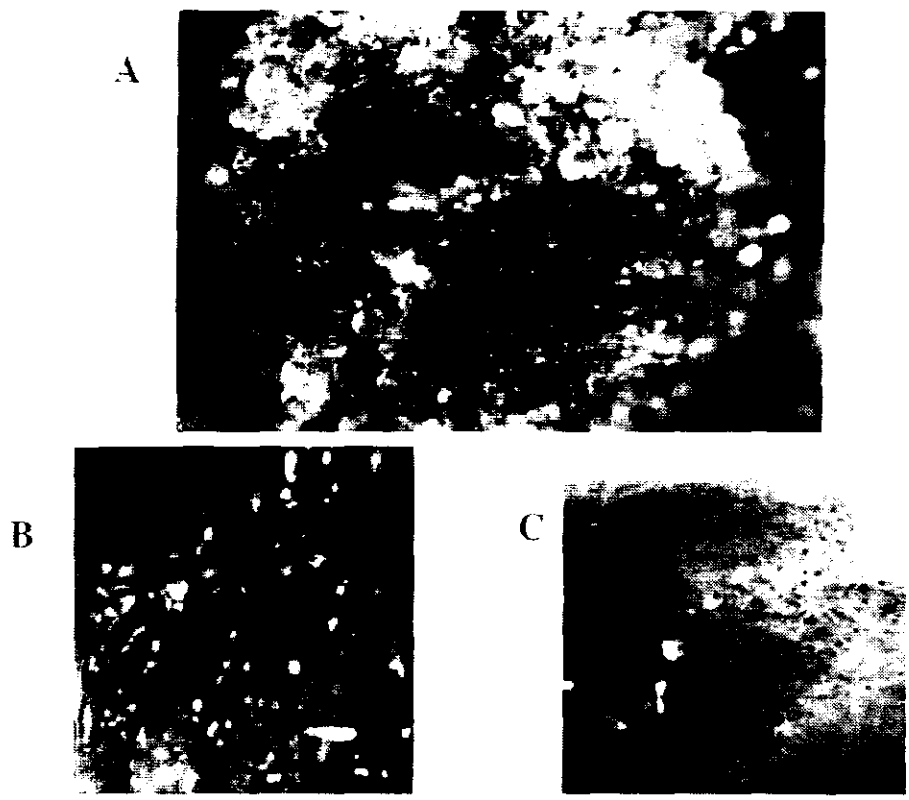
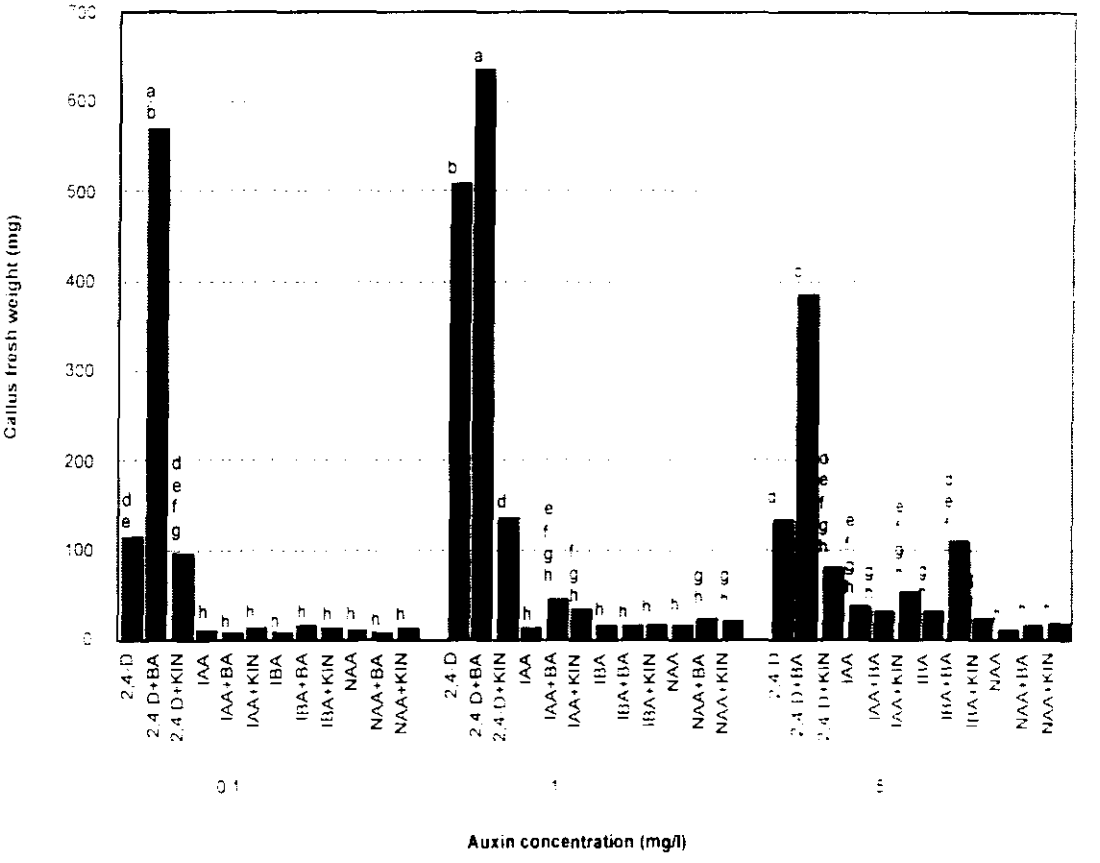


Fig. 10. Effects of growth regulators on callus proliferation from *H. [illegible]* hypocotyls explants following 8 weeks of culture in BRP medium.



Auxins were applied at the three concentrations indicated; cytokinins at 0.1 mg/l.
Means marked with the same letter are not significantly different according to the LSD test $p < 0.5$

Table 1. Regeneration of *H. persicum* after intense pruning (simulated browsing)

Parameter	Experimental plants				Control plants		
	year:						
	0. before pruning	0. end of growth	3	4	0. end growth	3	4
Height, cm	230	120	175	200	230	250	250
Length of one-year-old shoots, cm	35	50	55	55	35	40	35
Number of one-year-old shoots	400	480	550	650	360	350	360
Crown diameter, cm	160	90	130	150	250	255	250
Forage mass up to 120 cm, kg	0.4	2.0	3.0	4.0	0.5	0.4	0.3
Forage mass above 120 cm, kg	1.6	0.0	0.5	1.0	1.8	1.7	2.0
Total forage mass, kg	2.0	2	3.5	5	2.3	2.1	2.3

Table 2. Regeneration of *H. aphyllum* after intense pruning (simulated browsing)

Parameter	Experimental plants				Control plants		
	year:						
	0. before pruning	0. end growth	3	4	0. end growth	3	4
Height, cm	260	130	165	175	260	265	260
Length of one-year-old shoots, cm	30	50	65	60	35	37	35
Number of one-year-old shoots	213	400	1100	1100	360	355	350
Crown diameter, cm	230	120	150	170	230	235	230
Forage mass up to 120 cm, kg	1.0	5.0	6.0	6.5	1.0	1.3	1.0
Forage mass above 120 cm, kg	4.7	1.5	2.0	2.5	2.3	3.2	3.5
Total forage mass, kg	5.7	6.5	8	9.0	3.3	4.5	4.5

Table 3. Field performance of *Haloxylon aphyllum* and *H. persicum* seedlings planted at the Blaustein Institute.

observation date	ht. , cm. *H. aphyllum*	*H. persicum*
13.5.97	15-24	22-35
15.7.97	31-73	32-49
17.8.97	31-94	32-49
17.9.97	31-101	32-49
3.5.98	31-118	35-52
10.8.98	33-130	37-54

Table 4. Seed disinfestation

Disinfectant	Time, min.	Contamination, %
1.58% NaOCl	10	20% fungal
	20	5% fungal
	25	none
0.05% (w/v) $HgCl_2$	1.0	5% fungal
	1.5	none
	2.0	none

Table 5. Disinfestation of axillary shoots of three and four month old seedlings growing in a greenhouse and of a two-year-old plant growing in the field

Disinfectant	Time (min.	Contamination(%)
1.58% NaOCl (30% Commercial bleach)	15	100% fungal and bacterial contamination
	20	100% fungal and bacterial contamination
	30	Shoots browned, 80% bacterial contaminatio
0.05% $HgCl_2$	3	100% fungal contamination
	4	100% fungal and bacterial contamination
	5	100% fungal and bacterial contamination
0.1% $HgCl_2$	3.5	100% fungal and bacterial contamination
	4	100% fungal and bacterial contamination
	5	Shoots browned, 80% bacterial contaminatio

Table 6. Effects of BA +/- IAA on shoot induction from cotyledonary nodes of *H. aphyllum* cultured on full-strength MS medium

Growth regulators (mg l) IAA	BA	No. of buds and shoots per explant (Mean ± SE)	Percentage of explants producing buds and shoots	Shoot length (cm) (Mean ± SE)
0.0	0.0	0.00^{e}	0	0.00 ± 0.00
0.0	0.5	2.0 ± 0.00^{c}	100	0.12 ± 0.01
0.0	1.0	2.0 ± 0.00^{c}	100	0.12 ± 0.01
0.0	2.5	4.6 ± 0.56^{a}	100	0.16 ± 0.02
0.0	5.0	2.3 ± 0.30^{c}	100	0.10 ± 0.001
0.0	10.0	0.6 ± 0.22^{de}	50	0.10 ± 0.003
0.1	0.0	0.3 ± 0.15^{de}	30	0.09 ± 0.00
0.1	0.5	1.0 ± 0.25^{d}	70	0.10 ± 0.001
0.1	1.0	3.4 ± 0.30^{b}	100	0.11 ± 0.01
0.1	2.5	4.0 ± 0.33^{ab}	100	0.14 ± 0.02
0.1	5.0	0.5 ± 0.22^{de}	40	0.09 ± 0.004
0.1	10.0	0.2 ± 0.13^{e}	20	0.09 ± 0.00

*Mean values followed by the same superscript are not significantly different at $p < 0.05$ using LSD test.

Table 7. Effects of BA +/- IAA on shoot induction from cotyledonary nodes of *H. aphyllum* cultured on half-strength MS medium

Growth regulators (mg l) IAA	BA	No. of buds and shoots per explant (Mean ± SE)	Percentage of explants producing buds and shoots	Shoot length (cm) (Mean ± SE)
0.0	0.0	0.9 ± 0.31^{e}	50	0.10 ± 0.00
0.0	0.5	2.1 ± 0.10^{c}	100	0.10 ± 0.00
0.0	1.0	1.6 ± 0.16^{cd}	100	0.29 ± 0.03
0.0	2.5	3.3 ± 0.26^{a}	100	0.33 ± 0.04
0.0	5.0	1.5 ± 0.16^{cd}	100	0.28 ± 0.03
0.0	10.0	1.4 ± 0.16^{cde}	100	0.13 ± 0.02
0.1	0.0	0.0 ± 0.00^{f}	0	0.00 ± 0.00
0.1	0.5	1.1 ± 0.23^{de}	80	0.14 ± 0.02
0.1	1.0	1.2 ± 0.24^{de}	80	0.13 ± 0.02
0.1	2.5	1.8 ± 0.13^{bc}	100	0.13 ± 0.03
0.1	5.0	2.1 ± 0.23^{c}	100	0.10 ± 0.001
0.1	10.0	2.8 ± 0.24^{a}	100	0.10 ± 0.09

*Mean values followed by the same superscript are not significantly different at $p < 0.05$ using LSD test.

Table 8. Effects of BA + - IAA on shoot induction from cotyledonary nodes of *H. aphyllum* cultured on BRP medium

Growth regulators (mg l)		No. of buds and shoots per explant (Mean ± SE)	Percentage of explants producing buds and shoots	Shoot length (cm) (Mean ± SE)
IAA	BA			
0.0	0.0	1.6 ± 0.22^{a}	90	0.28 ± 0.04
0.0	0.5	1.1 ± 0.31^{abc}	60	0.10 ± 0.00
0.0	1.0	0.8 ± 0.24^{bc}	60	0.10 ± 0.00
0.0	2.5	0.5 ± 0.22^{c}	40	0.10 ± 0.00
0.0	5.0	1.1 ± 0.23^{abc}	80	0.14 ± 0.02
0.0	10.0	1.0 ± 0.25^{abc}	70	0.11 ± 0.01
0.1	0.0	1.3 ± 0.26^{ab}	80	0.11 ± 0.01
0.1	0.5	1.4 ± 0.22^{ab}	90	0.11 ± 0.01
0.1	1.0	1.4 ± 0.33^{ab}	70	0.11 ± 0.01
0.1	2.5	1.2 ± 0.32^{abc}	70	0.11 ± 0.01
0.1	5.0	1.0 ± 0.25^{abc}	70	0.13 ± 0.02
0.1	10.0	1.1 ± 0.23^{abc}	80	0.12 ± 0.02

*Means followed by the same superscript are not significantly different at $p = 0.05$ using LSD test.

Table 9. Effects of BA + - IAA on shoot induction from axillary shoots of *in vitro* seedlings of *H. aphyllum* cultured on full-strength MS medium

Growth regulators (mg l)		No. of buds and shoots per explant (Mean ± SE)	Percentage of explants producing buds and shoots	Shoot length (cm) (Mean ± SE)
IAA	BA			
0.0	0.0	1.5 ± 0.34^{abc}	90	0.10 ± 0.001
0.0	0.5	1.4 ± 0.26^{abcd}	80	0.10 ± 0.001
0.0	1.0	2.0 ± 0.36^{a}	90	0.16 ± 0.02
0.0	2.5	1.4 ± 0.30^{abcd}	70	0.10 ± 0.00
0.0	5.0	0.9 ± 0.31^{cdef}	60	0.10 ± 0.002
0.0	10.0	0.8 ± 0.24^{cdef}	60	0.09 ± 0.006
0.1	0.0	0.2 ± 0.13^{f}	20	0.09 ± 0.00
0.1	0.5	0.6 ± 0.22^{def}	50	0.10 ± 0.00
0.1	1.0	2.2 ± 0.41^{a}	90	0.19 ± 0.01
0.1	2.5	1.2 ± 0.24^{bcde}	80	0.10 ± 0.00
0.1	5.0	0.9 ± 0.27^{cdef}	60	0.10 ± 0.002
0.1	10.0	0.4 ± 0.22^{ef}	30	0.10 ± 0.003

*Means followed by the same superscript are not significantly different at $p = 0.05$ using LSD test.

Table 10. Effects of BA + - 0 IAA on shoot induction from axillary shoots of *in vitro* seedlings of *H.* aphyllum cultured on half-strength MS medium

Growth regulators (mg l)		No. of buds and shoots per explant (Mean ± SE)	Percentage of explants producing buds and shoots	Shoot length (cm) (Mean ± SE)
IAA	BA			
0.0	0.0	0.0 ± 0.00^{c}	0	0.00 ± 0.00
0.0	0.5	1.2 ± 0.20^{abc}	90	0.10 ± 0.00
0.0	1.0	1.7 ± 0.21^{a}	100	0.10 ± 0.00
0.0	2.5	0.7 ± 0.26^{bcd}	50	0.10 ± 0.00
0.0	5.0	1.3 ± 0.30^{ab}	80	0.10 ± 0.001
0.0	10.0	1.2 ± 0.32^{abc}	70	0.10 ± 0.002
0.1	0.0	0.0 ± 0.00^{c}	0	0.00 ± 0.00
0.1	0.5	0.6 ± 0.22^{cde}	50	0.10 ± 0.00
0.1	1.0	1.1 ± 0.23^{abc}	80	0.25 ± 0.04
0.1	2.5	1.7 ± 0.15^{a}	100	0.11 ± 0.01
0.1	5.0	1.0 ± 0.25^{bcd}	70	0.10 ± 0.003
0.1	10.0	0.4 ± 0.22^{de}	30	0.10 ± 0.003

*Means followed by the same superscript are not significantly different at $p > 0.05$ using LSD test.

Table 11. Effects of BA + - IAA on shoot induction from axillary shoots of *in vitro* seedlings of *H.* aphyllum cultured on BRP medium

Growth regulators (mg l)		No. of buds and shoots per explant (Mean ± SE)	Percentage of explants producing buds and shoots	Shoot length (cm) (Mean ± SE)
IAA	BA			
0.0	0.0	0.0 ± 0.00^{c}	0	0.00 ± 0.00
0.0	0.5	0.4 ± 0.22^{ab}	30	0.10 ± 0.00
0.0	1.0	0.5 ± 0.26^{ab}	30	0.10 ± 0.00
0.0	2.5	0.7 ± 0.30^{ab}	40	0.15 ± 0.03
0.0	5.0	1.1 ± 0.27^{a}	70	0.13 ± 0.02
0.0	10.0	0.7 ± 0.30^{ab}	40	0.15 ± 0.03
0.1	0.0	0.6 ± 0.26^{ab}	40	0.12 ± 0.03
0.1	0.5	0.5 ± 0.22^{ab}	40	0.12 ± 0.02
0.1	1.0	1.0 ± 0.29^{a}	60	0.17 ± 0.02
0.1	2.5	1.0 ± 0.25^{a}	70	0.15 ± 0.02
0.1	5.0	0.7 ± 0.26^{ab}	50	0.12 ± 0.02
0.1	10.0	0.8 ± 0.29^{a}	50	0.14 ± 0.02

*Means followed by the same superscript are not significantly different at $p > 0.05$ using LSD test.

Table 12. Effects of BA + - IAA on shoot induction from cotyledonary nodes of *H. aphyllum* cultured on full-strength MS medium

Growth regulators (mg l)		No. of buds and shoots per explant (Mean ± SE)	% explants producing buds and shoots	Shoot length (cm) (Mean ± SE)
IAA	BA			
0	2.5	3.4 ± 0.47[a]	100	0.13 ± 0.02
0.001	2.5	3.6 ± 0.42[a]	100	0.16 ± 0.02
0.01	2.5	3.3 ± 0.33[a]	90	0.15 ± 0.02[a]
0.1	2.5	4.0 ± 0.21[a]	90	0.18 ± 0.02[a]

*Means followed by the same superscript in each column are not significantly different at p = 0.05 using LSD test.

Table 13. Effects of BA + - IAA on shoot induction from axillary shoots of *in vitro* seedlings of *H. aphyllum* cultured on full-strength MS medium

Growth regulators (mg l)		No. of buds and shoots per explant (Mean ± SE)	% explants producing buds and shoots	Shoot length (cm) (Mean ± SE)
IAA	BA			
0	1.0	1.7 ± 0.33[a]	90	0.16 ± 0.02[a]
0.001	1.0	1.9 ± 0.17[a]	100	0.12 ± 0.01[a]
0.01	1.0	1.9 ± 0.27[a]	90	0.13 ± 0.02[a]
0.1	1.0	1.8 ± 0.35[a]	90	0.13 ± 0.02[a]

*Means followed by the same superscript in each column are not significantly different at p = 0.05 using LSD test.

Table 14. Rooting of axillary shoots from *in vitro* seedlings of *H. aphyllum* following pre-incubation in IAA-containing BRP medium and culture on hormone-free BRP medium for 30 days.

IAA (mg l)	Pre-incubation in hormonal medium							
	7days		10 days		15 days		20 days	
	Shoots rooted (%)	Root length(cm) (mean ±SE)	Shoots rooted (%)	Root length(cm) (mean ±SE)	Shoots rooted (%)	Root length(cm) (mean ±SE)	Shoots rooted (%)	Root length(cm) (mean ±SE)
0	-	-	-	-	-	-	-	-
0.5	-	-	-	-	50	0.4±0.06	-	-
1.0	-	-		-	30	0.5±0.09		-
2.5	-	-	40	0.5±0.09	30	0.5±0.09	30	0.2±0.0
5.0	-	-	50	1.2±0.09	50	0.8±0.09	-	-
10.0	-	-	50	1.0±0.04	30	0.3±0.09	-	-
15.0	-	-	-	-	-	-	-	-
20.0	30	0.5±0.15	-	-			-	-

Table 15. Callus induction from zygotic embryos of *H. aphyllum*.

2,4-D (mg/l)	BA (mg/l)	% Explants with callus	Relative callus formation
1.00	0.50	100	+
2.00	1.00	100	++
4.00	2.00	60	+
8.00	4.00	50	+
16.00	8.00	20	+
0	0	0	-

*Relative amount of callus: + = 5 mm^2; - = no callus formation

Table 16. Callus induction from seedling explants under light and dark conditions in MS and BRP media.

Illumination	Explant	Medium	% Explants with callus	Relative callus formation *
light	hypocotyl	MS	100	+
light	cotyledon	MS	100	+
dark	hypocotyl	MS	100	+
dark	cotyledon	MS	100	+
light	hypocotyl	BRP	100	+++
light	cotyledon	BRP	100	++
dark	hypocotyl	BRP	100	++
dark	cotyledon	BRP	100	++

Growth regulators: 2,4-D + BA (2.00+1.00 mg/l) in each treatment
*Relative amount of callus: + = 5 mm^2

Table 17. Callus induction and proliferation from seedling explants with and without illumination during two culture cycles.

Treatment	Illumination	Explant	Duration of phase I of culture	% Explants with callus	Relative callus formation in phase I*	Callus increase during phase II of culture*
1	-	Hypocotyl	5 days	60	-	.
2	+	Hypocotyl	10 days	80	-	
3	+	Hypocotyl	15 days	100	- -	-
4	-	Hypocotyl	20 days	100	- - -	- -
5	+	Hypocotyl	30 days	100	- - -	- -
6	+	Cotyledon	5 days	70	-	.
7	+	Cotyledon	10 days	90	-	
8	+	Cotyledon	15 days	100	- -	-
9	+	Cotyledon	20 days	100	- -	-
10	+	Cotyledon	30 days	100	- - -	- -
11	-	Hypocotyl	5 days	70	+	.
12	-	Hypocotyl	10 days	90	+	-
13	-	Hypocotyl	15 days	100	- -	- -
14	-	Hypocotyl	20 days	100	- -	- -
15	-	Hypocotyl	30 days	100	- - -	- -
16	-	Cotyledon	5 days	60	-	-
17	-	Cotyledon	10 days	80	-	-
18	-	Cotyledon	15 days	100	-	-
19	-	Cotyledon	20 days	100	- -	-
20	-	Cotyledon	30 days	100	- -	-

*Relative amount of callus: + = 5 mm ^{2}; - = no callus formation

Table 18. Callus induction and proliferation from hypocotyls with combinations of 2,4-D and BA or Kin during two culture cycles

Treatment	Phytohormone combination	% Explants with callus	Relative callus formation*	Callus increase*
	2,4-D+BA (mg l)			
1	0.1 + 0.1	100	+	+
2	0.5 + 0.1	100	++	++
3	1.0 + 0.1	100	++++	++++
4	2.0 + 0.1	100	+++	++
5	5.0 + 0.1	100	+	++
6	0.1 + 1.0	100	++	+
7	0.5 + 1.0	100	++	+
8	1.0 + 1.0	100	+	++
9	2.0 + 1.0	100	+++	++
10	5.0 + 1.0	100	+	+
	2,4-D+Kin (mg l)			
11	0.1 + 0.1	100	+	+
12	0.5 + 0.1	100	+	++
13	1.0 + 0.1	100	++	++++
14	2.0 + 0.1	100	++	+
15	5.0 + 0.1	100	+	+
16	0.1 + 1.0	100	++	+
17	0.5 + 1.0	100	+	+
18	1.0 + 1.0	100	+	+
19	2.0 + 1.0	100	++	++
20	5.0 + 1.0	100	+	+

*Relative amount of callus + = 5 mm²; - = no callus formation

IMPACT RELEVANCE AND TECHNOLOGY TRANSFER

Livestock production on rangeland is an important and widespread component of the rural economy of Turkmenistan. Work on identification of the best fodder shrubs and development of appropriate techniques for their cultivation has been underway over the course of many years at several experiment stations associated with the Desert Research Institute of Turkmenistan and by its research staff. That work was integrated with this research program. Members of the DRI-T participated in all aspects of the research in their home country as well as at the Blaustein Institute and the lines of communication between the groups remain open.

The information on water harvesting, seedling preparation, planting and cultivation, developed at the DRI-T, is already being implemented in Turkmenistan. The method developed for seed storage at low temperature can be implemented at the DRI-T, to establish a seed repository of *Haloxylon*, to meet its own and local needs as well as to share seed with other institutions and countries. Specific equipment and supplies for working in tissue culture have been purchased for the DRI-T. The techniques of micropropagation have been taught to members of the DRI-T staff, they have had significant hands-on experience. They have the ability to implement the protocol developed for micropropagation of *H. aphyllum* and to work in tissue culture on other crops that might be of value to their country. We are prepared to share progress (application of the procedures to adult plant material) with them in the future, as we, at the BIDR, progress.

The program has had direct impact on six members of the DRI-T faculty, Prof. N. Orlovsky, Prof A.G. Babaev, Dr. M. Nikolaev, Dr. Moukhamet Dourikov, Dr. Olga Antsiferova and Ms. Nabat Mamedova, who spent various periods of time at the BIDR during the course of this program. Orlovsky, who was deputy director of the DRI-T and the senior Turkmen investigator in this program, came to the BIDR in 1995. In 1997 he received a special appointment to the faculty of the Blaustein Institute. Throughout that period and since, he has worked on aspects of this research program. Babaev, director of DRI-T during the course of this program (subsequently retired) visited the BIDR, Ben-Gurion University and various

other organizations and institutions in Israel, giving him a better understanding of this country, its agricultural production capacity and approaches to solving problems through research and development. Nikolaev, Dourikov, Antsiferova and Mamedova each spent extended periods at the BIDR, participating in the research as well as attending courses. In addition to the research experience, their stays here contributed significantly to each's level of spoken and written English and understanding of modern approaches to research.

PROJECT ACTIVITIES/OUTPUTS

Training and Visits of Senior Scientists

Dr. M. Nikolaev of DRI-T spent 22 months at the Blaustein Institute, from February 1995 through November 1996, working on clonal propagation.

Prof. N. Orlovsky joined the research group at the Blaustein Institute in November 1995, working on seed storage, field studies and greenhouse trials of the effects of salinity.

E. Birnbaum and N. Orlovsky visited Turkmenistan in March 1996, November 1996, November 1998 and May 2000. Each trip included time at The Desert Research Institute in Asegabat as well as field trips to Sely, Repetec and Karakul, research stations operated by the institute. During the second visit appropriate rooms at DRI-T were identified to house the tissue culture laboratory and its layout planned.

Prof. A.G. Babaev, Director of the Desert Research Institute of Turkmenistan visited the Blaustein Institute in May 1996.

Dr. Olga Antsiferova and Ms. Nabat Mamedova of DRI-T were both at the Blaustein Institute for 20 months, from September 1996 to April 1997, working on tissue culture aspects of the project and learning methodology.

Dr. Moukhamet Dourikov visited the Blaustein Institute in February, 1999.

Publications and Presentations at International Congresses

Birnbaum. E.B. 1996. Pastureland Degradation and Revegetation. Invited Speaker. National Conference on Combating Desertification. Aschabat. Turkmenistan.

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Parveen and E.H. Birnbaum. 2001. *In vitro* propagation of *Haloxylon aphyllum* (Minkw.) Iljin. Acta Hort 560. ISHS:461-464.

Poster presented at the Fourth International Symposium on In Vitro Culture and Horticultural Breeding. Tampere. Finland. 2-7 July. 2000.

Parveen. 2000. Clonal Propagation of *Haloxylonaphyllum* (Minkw.) Iljin: Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science by Ms.

Ramirez. A.E. and E.H. Birnbaum. 2001. Induction and characterization of callus in *Haloxylon aphyllum* (Minkw.) Iljin. Acta Hort 560:465-468.

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Ramirez. Aida Eleonora. 2000. Induction of Somatic Embryogenesis in *Haloxylon aphyllum*: Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science.

PROJECT PRODUCTIVITY

The research objectives of the program were a) to develop a protocol for seed storage, b) to study the phenology of the species and to define the climatic parameters of the regions of their distribution, c) to select superior *Haloxylon* shrubs in field trials conducted in Turkmenistan and *in vitro*, and d) to develop systems for mass propagation of selections by rooting cuttings, micropropagation and somatic embryogenesis.

a) Seed lots with high initial rates of germination lost viability over time when stored at room temperature in the dark, but could be stored for extended periods (at least 7 years, as determined at the time of this report) in darkness at –4o C with little loss of viability. During the research we identified seed lots that had high rates of germination shortly after harvest and lots with low initial germination rates. These differences were probably due to environmental conditions during seed development and or genetics. The causes of differences between seed lots were not investigated, and storage trials were conducted only with the better seed lots.

b) Information on the phenology of H. aphyllum and H. persicum was assembled through an in depth review of the literature (largely Russian-language) on these species, collection of data in previous years at the research stations of the DRI in Turkmenistan, and trials during this program at those research stations as well as at the BIDR in Israel. The report characterizes each species, its preferred ecological niche, and its response to severe cutting back.

c) Selection of superior adult *Haloxylon* shrubs was an integral part of the field trials conducted in Turkmenistan. Seed used in the *in vitro* studies were derived from these selections. The two species, *H. aphyllum* and *H. persicum*, differed in their responses *in vitro*: the former showing significantly greater shoot extension than the latter during the stage of axillary shoot extension. Differences among clones of each species were noted too with respect to shoot elongation, serving as a key trait for *in vitro* selection. Clones that demonstrated more rapid and greater shoot extension were selected, as such were more amenable to successful post-rooting acclimatization.

d) Work was conducted on development of three systems for mass clonal propagation: rooting of cuttings, micropropagation and somatic embryogenesis. A complete micropropagation system was developed, using explants taken from seedlings germinated *in vitro*. The work on rooting cuttings was abandoned early because of failures in controlling environmental conditions. Appropriate culture media, conditions and sequence of treatments were defined for generation and multiplication of putative proembryonic callus from explants that originated in somatic embryos of *H. aphyllum*. Spherical and elongated (suspensor-like) cell types, associated with embryogenic cultures, were generated, in this study. Cell clusters differentiated into white bodies with deep green meristematic centers. Actual somatic embryos were not identified, but the similarity of the cell types and tissue organization in our cultures to embryogenic calli of other species that have been described, lead us to believe that this tissue holds the potential to produce somatic embryos.

FUTURE WORK

Work in several of the areas addressed in this program should continue.

Superior shrub selection in such long-lived species must be viewed as a long-term objective, to be carried out with promising adult specimens as well as with populations generated vegetatively from selections. Selection should continue in Turkmenistan and other Central Asian republics, in Israel and the Middle East and in countries where fodder production on ASAL rangeland is important.

Systems for rooting cuttings and for initiating tissue cultures from adult plants for micropropagation should be developed. Both will be important tools for cloning selections made in field trials, which will enable large-scale planting of superior material.

Development of the system of somatic embryogenesis should be completed. This will provide the lowest-cost system of clonal propagation of these species.

Some aspects of the work on *Haloxylon* will be addressed in the AID CDR program, AID CDR-C16-091, Establishment of Productive Rangeland with High-Yielding Fodder Shrubs, a collaboration now underway with scientists at the University of Pretoria in South Africa. We hope financial resources will be found to pursue other aspects with our Turkmen collaborators.

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