PRELIMINARY STUDIES ON *IN VITRO* CULTURE OF *ATRIPLEX HORTENSIS* L.

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ABSTRACT

Atriplex hortensis L. commonly Known as Vust-e-Haak was regenerated from *in vitro* raised seedlings on MS ½ medium. Callus lines were induced from *in vitro* raised nodal segments and shoot tips explants on MS medium supplemented with BAP. Shoot tips and nodal segments when cultivated on combination NAA 7.5 μM+BAP 7.5 μM induced enhanced axillary branching. However, only elongation of main shoot and no axillary branching of shoot was observed on NAA (12.5μM) + BAP (12.5μM) from the nodal segment and shoot tips explants.
Keywords: *Atriplex hortensis,* shoot tips, nodal segments, callus, axillary branching
Abbreviations: MS- Murashige and Skoog; BAP-6–benzylaminopurine; NAA-α napthaleneacetic acid

INTRODUCTION

Atriplex species are members of the chenopods and include both C_3 and C_4 plants. The genus is quite variable and widely distributed. It includes many desert and seashore plants and halophytes, as well as plants of moist environment. There are more than 200 hundred species, most of which are highly tolerant to drought and salt. *Atriplex* species take up NaCl from saline soil and sequester it into the salt glands in their leaves. This is a useful characteristic and allows them to be used for revegetation in saline or arid /semi arid lands. *Atriplex* species contain high level of protein. Many species of the genus *Atriplex* are edible and are also excellent for livestock because of their favorable crude protein content (Mc Kell, 1994). However, the favored species for human consumption is *A.hortensis* (Garden Orache). The Garden Orache, also called Red Orache, Mountain Spinach, or French spinach, is an annual leaf vegetable with salty, spinach- like taste. *Atriplex hortensis* L. commonly called as vust-e- haak in Kashmir belongs to family Chenopodiaceae. It is widely grown in India and is much used for consumption in Kashmir.

Genetic transformation of *Atriplex* would be valuable for molecular farming and the production of the economically valuable proteins in saline and semi arid areas otherwise useless for agricultural crop production. It is in this direction that a preliminary attempt has been made to generate multiple shoots for successful micro propagation in *Atriplex hortensis* using nodal segments and shoot tips explants from *in vitro* raised seedlings plants.

MATERIAL AND METHODS

Seeds of *Atriplex hortensis* were washed with lab. detergent (Cedpol) containing a few drops of Tween-20 under running tap water for 5-10 minutes. These washed seeds after overnight soaking in distilled water, were surface the presence of all kinds of water-borne pathogens, testing classical microbial indicators of pollution (total coliforms, fecal coliforms and fecal streptococcus) have been extensively used (Feacham, 1980; Bhathacherjee, 1986; WHO, 1993; Schaffter and Parriaux, 2002; Celico *et al.*, 2004). The indicator bacteria are not dangerous, but their presence indicates fecal contamination (WHO, 2004).

In Anantnag, the surface water supplies are dwindling due to global warming, deforestation, large scale growth in population and settlements with out commensurate improvement of infrastructure and civic amenities, the dependency on water from natural springs is increasing. As always is the consequence of ill-planned anthropogenic activities, surface water resources were first to be affected. A concrete box (muddy box at a few locations) is constructed around most of the spring sources and a proportion of the water is then supplied to local population via pipes. Although the springs have served as traditional water sources for the inhabitants for centuries, but little attention is given for their cleaning and development. As the springs are mostly in remote areas, due to the lack of awareness of water quality the families living nearby use the springs for bathing, washing and other domestic purposes and the government hardly bothers.

The fact that the very existence of most parts of Anantnag district depend on these springs necessitated this study, the first of its kind, in the Kashmir valley. The present study aims to investigate the water quality of the 40 perennial springs with a focus on heavy metals and classic microbial indicators.

MATERIAL AND METHODS

Description of the Study Area

Anantnag area lies towards the SE of NW-SE trending intermontane valley of Kashmir, is bounded by Great Himalayan Range towards NE and Pir Panjal Range towards SW. The bowl shaped valley is 135 km long with the maximum width being 40 km. Its floor stands 1585 to 1900 m above the mean sea level in the flood plain of river Jhelum. The river Jhelum flows through the valley and has its origin through various spring discharges and a number of streams fed by glaciers in high mountains. Most of the springs of Kashmir Valley are concentrated in Anantnag. The place name literally means numerous springs in the Kashmiri language. The area lies between $33^0\,20'$ N to $34^0\,15'$ N latitudes and $74^0 30'$ E to $75^0\,35'$ E longitudes, cover an area of about 3984 km^2 (Fig. 1) with a population of about 11, 72,434 as per 2001 census. In addition, hundreds of thousands of pilgrims pass through this area to visit the holy Amarnath cave in summer. The area experiences temperate climate with an average annual rainfall of about 1200 mm.

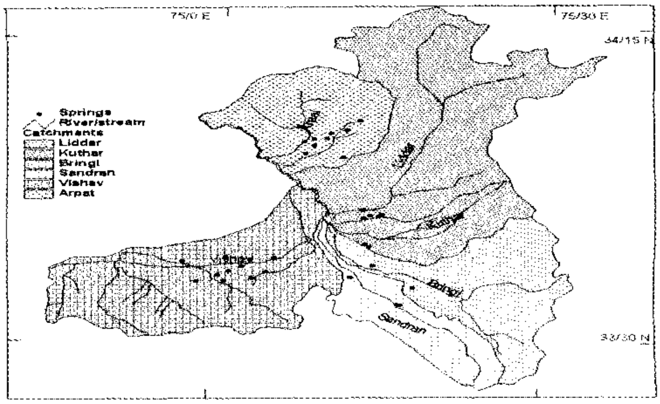

Fig. 1. Location map showing springs in and around Anantnag

Geological and Hydrogeological Setup

Paleozoic sedimentaries, Triassic Limestone, Karewas and alluvium are the predominant formations of the area with limestones, volcanics, shales, sandstones and unconsolidated sediments as the dominant lithologies (Bhat, 1989; Wadia, 1976). The higher altitude regions (> 3000 m above mean sea level), the recharge areas, are mostly occupied by the Panjal Traps and the Triassic Limestone. The Valley is filled up with a great thickness (> 2000 m) of fluviolacustrine sediments of Quaternary age belonging to the Karewa Group and the river deposited alluvium of recent age. The bed rock is either Triassic Limestone or Panjal Traps (Datta, 1983; Ganju and Khar, 1984).

Four types of springs were found: karst, alluvial, karewa and warm springs (Jeelani, 2005). Joints and fractures, obviously, provide the most significant structural control for the very existence of the springs in the Anantnag area. These planes establish a hydrological continuity between the recharge zones at higher altitudes and the discharge sites at the lower altitudes and valley floor. Two spring groups were identified: one group towards the Great Himalayan Range and the other towards the Pir Panjal Range. All the springs have their reservoirs mainly in Triassic Limestone or Panjal Traps (Jeelani, 2004) Spring discharges were highly variable from about 5 L/s to 1880 L/s. In general, karst springs have higher discharges (~1000 L/s) and warm springs lower (~1 L/s).

Three sets of water samples were collected from all the 40 perennial springs in summer 1999, two sets in polyethylene bottles (one set acidified for trace elements) and one set for bacteriological analyses in clean, airtight and sterile glass bottles (transported in a refrigerated box). To avoid floating debris the samples were taken at a depth of > 15 cm. The samples were analyzed for physico-chemical parameters using the techniques of (APHA, 1992). Trace elements were determined using atomic absorption spectrophotometer (AAS). For bacteriological study the samples were analysed for classic indicator bacteria: total coliform, fecal coliform and faecal streptococci. For the detection and enumeration of indicator bacteria in water, multiple-tube fermentation technique was followed, in which measured volumes of water are added to replicate tubes of a suitable liquid medium, following standard methods of (APHA, 1992).

RESULTS AND DISCUSSION

The summary of results of physico-chemical analysis (Table 1) shows that the spring waters were feebly acidic to alkaline with low total dissolved solids (TDS).

Table 1. Summary of results of physico-chemical and bacteriological analyses of Anantnag springs

Parameter with unit	Range observed in two sets of samples	Acceptable and maximum permissible limit in drinking water (WHO, 1993)
pH	6.5-7.6	6.5-8.5
TDS (mg/L)	100-256	1000
Ca^{2+}(mg/L)	8-38	75-200
Mg^{2+}(mg/L)	2-20	50
Na^{+}(mg/L)	trace-54	200
K^{+}(mg/L)	trace-10	12
HCO_3^{-}(mg/L)	38-270	—
SO_4^{2-}(mg/L)	1-63	200-400
Cl^{-}(mg/L)	3-17	250
NO_3^{-}(mg/L)	trace-8	10-50
F^{-} (mg/L)	0.02-1.1	1.0-1.5
Fe (ppm)	0.08-0.51	0.3
Pb (ppm)	0.002-0.02	0.05
Zn (ppm)	0.1-2.57	5
Cd (ppm)	n.d-0.002	0.005
Cr (ppm)	0.04-0.06	0.05
Cu (ppm)	0.01-0.1	1
Mn (ppm)	n.d-0.1	0.1
Total coliform (MPN/100mL)	13-260	0
Fecal coliform (MPN/100mL)	2-92	0
Fecal streptococci (MPN/100mL)	3-15	0

The concentration of all the major ions that give first hand idea about the suitability of water for different purposes, in all the spring waters was within the permissible limits given by World Health Organisation (WHO, 2004) for drinking purposes. The concentration of a set of seven heavy metals, Fe, Zn, Pb, Cu, Cd, Mn and Cr, were also determined in the water samples. The concentration of Pb (0.002 – 0.02 ppm), Zn (0.1 – 2.5 ppm), Cu (0.01 – 0.1 ppm), Cd (nd – 0.002 ppm) and Mn (0-0.1 ppm) is found to be within WHO permissible limits (Table 1). However, the concentration of Fe (0.08 – 0.51 ppm) and Cr (0.04 – 0.06 ppm) of most of the spring water samples exceeded the prescribed WHO limit of 0.3 ppm and 0.05 ppm, respectively. Within the permissible levels of drinking water quality standards the heavy metals are essential in the human and animal metabolism and the excessive concentrations are toxic. The concentration of the heavy metals in fresh surface and groundwater are generally small. The higher concentrations were found to be caused due to anthropogenic activities and industrial wastes. As the area under investigation is free from industries, the sources of heavy metals may be considered as lithogenic not anthropogenic with Zn as the only exception. Dissolved Zn concentrations in relatively undisturbed water bodies typically range between 10^{-9} – 10^{-8} mol/kg (Shiller and Boyle, 1985). The high and variable concentration of Zn (2 - 38 × 10^{-6} mol/kg), although within the drinking water quality standards is related to the fertilizer application in the area, as ZnS is a common component of the fertilizers used for rice cultivation. Besides, the springs close to the agricultural fields show higher concentrations. Iron being second most abundant metallic element in the earth's outer crust, is present in some of the minerals of igneous and metamorphic rocks and occur as a relatively abundant impurity in carbonate and sedimentary rocks (Hem, 1985). The iron may be scavenged from the hosted carbonate rocks (Triassic Limestone), basalts (Panjal Traps) and sediments (Karewas). As chromium is found in most of the soils (Bouwer, 1978), its presence in spring waters is attributed to the leaching from the soils. Environment Protection Agency (EPA) has set maximum contaminant level (MCL) for chromium at 0.1 ppm (http://www.epa.gov/) which is above the Cr concentration found in the spring waters. Besides, chromium (Cr^{6+}, more toxic, is reduced to Cr^{3+}, less toxic, in natural environment) is not acutely toxic to humans (Moore and Ramamoorthy, 1984) and do not pose any threat to humans who consume this water.

The quality assessment of water with reference to individual parameter gives an idea of the pollution with respect to the quality of the parameter under consideration. However, the overall quality of water with respect to heavy metals can be determined by quality indices i.e., composite influence of all the parameters. Evaluation of heavy metal pollution index (HPI) has taken wide attention in surface and groundwater used for drinking purposes (Horton, 1965; Joung *et al.*, 1979; Nishidia *et al.*, 1982; Tiwari and Mishra, 1985; Mohan *et al.*, 1996; Prasad and Bose, 2001). The heavy metal pollution index determined for all the spring waters in Anantnag is

10.07, which is far below the critical index value of 100. Hence, the waters that show higher concentration of some individual parameters are not overall contaminated with respect to heavy metal pollution.

The concentration of coliform bacteria in all the spring waters and types (Table 1) was well above the drinking water quality standards. The concentration of total coliforms was higher in all the spring types; karst springs (13 to 260 MPN/100 mL), karewa springs (161 to 240 MPN/100 mL), alluvial springs (92 to 260 MPN/100 mL) and warm springs (260 MPN/100 mL). The concentration of fecal coliform and fecal streptococci bacteria of the spring waters ranged from 2 to 92 MPN/100 mL and 1 to 18 MPN/100 mL, respectively. The concentration of the coliform bacteria was found to be lowest in the Kokernag spring, which may be attributed to its aloofness to the population. It is interesting to note that a positive correlation was found among the indicator bacteria and between total coliform bacteria and NO_3 concentration (Fig. 2 except karewa springs) indicating common source. The higher concentration of NO_3 in karewa springs is attributed to the use of fertilizers and pasture land, as these springs generally occur within pasture and rice cultivated lands where local people and cattle herds directly contaminate the water.

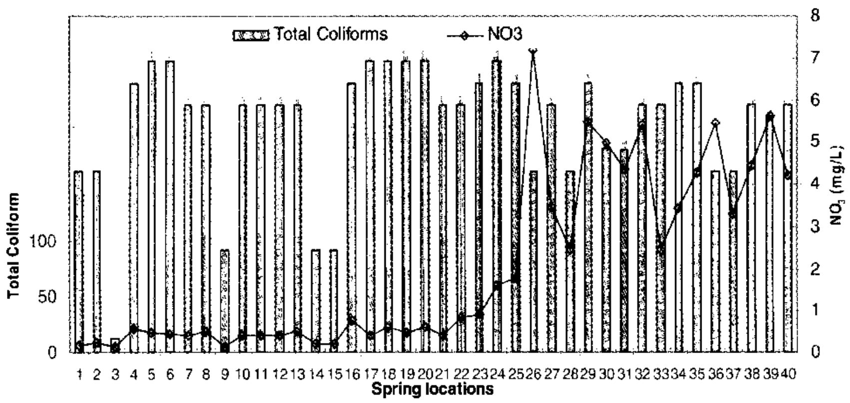

Fig. 2. Correlation of coliform bacteria with NO_3 in the spring waters

Although the relationship between the hydrogeological/geological characteristics of aquifers and bacterial population/extent of bacterial contamination of groundwater has received considerable attention (Henry *et al.*, 1987; Yates, 1990; Thorn and Coxon, 1992), the bacterial contamination was found to be equally severe in all the spring types (Fig. 3) irrespective of lithology through which they emerge.

The springs in carbonate rocks are more vulnerable to microbiological contamination due to karst related conduits which facilitate dissipation of contaminants easily. In rural areas pasture and manure spreading or open defecation nearer to springs often produces microbial contamination. The microbial contamination in most of the karst springs of Anantnag is attributed to such practices.

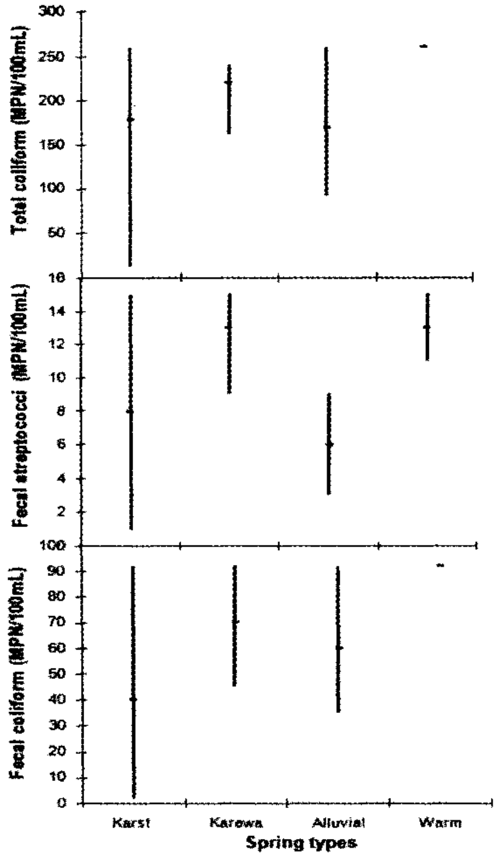

Fig. 3. Concentration of classic indicator bacteria (total coliform, fecal coliform, fecal streptococci) in water samples of different spring types showing the range and average

Besides the boxes constructed around the spring sources are not cleaned at all that further enhances the problem. However, the bacterial contamination in the springs (alluvial and karst springs) that are within the towns was found to be due to poor sanitation systems. The warm springs, in all likelihood, are most affected by bacterial contamination because of their maximum utilization in spite of relatively small discharge of <1 to 20 L/s. As for as Karewa springs are concerned, they generally occur within pasture lands and meadows where local people and cattle herds directly contaminate the water.

The most significant and alarming feature of the study is the persistence of bacterial contamination in all the springs. Higher level of inferred fecal contamination, though not surprising, is definitely disturbing and seems to highlight the necessity of protecting these precious water resources and their aquifers. The results have unequivocally demonstrated that the bacterial count in springs depends more on the proximity of the spring to anthropogenic activity rather than the lithology through which it emerges. As the depth to water table hardly exceeds 11 m in the summer, fecal contamination through local sources would continue to be the major cause of bacterial contamination unless some drastic remedial measures are initiated without any delay. The use of this untreated water could cause pathogenic water-borne diseases. Although the data related to water-borne diseases in the area is not available, there were cases of jaundice, hepatitis A and E, diarrhea, dysentery, and respiratory infection in children, gastroenteritis, viral fever and skin diseases.

CONCLUSIONS

Among the quality parameters some chemical (Fe and Cr) and microbiological parameters were found to be unsatisfactory in spring waters of the area and exceed permissible limit suggested by World Health Organisation (WHO, 1993). The spring water of the study area, therefore, was not safe for drinking purposes, unless the water is treated properly. The study also revealed the lithogenic source of high concentration of Fe and Cr. Poor sanitation system around the springs, lack of cleanliness of the spring boxes, open defecation and pasture lands were found to be the main causes of microbiological contamination.

The spring water should be treated before drinking. The sanitation system of the area must be improved and the cleanliness of the springs should be maintained on regular basis. The awareness programs on hygiene and spring maintenance should be arranged in the area.

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sterilized by 0.1% $HgCl_2$ for 15 minutes and finally rinsed 3-4 times with autoclaved double distilled water. Sterilized seeds were inoculated on MS ½ (1962) medium supplemented with 30 gm/l of sucrose. pH of the medium was adjusted to 5.8 and 0.8% agar was used as jelling agent. The cultures were maintained at 23 ±2^{0}C with 55-65% relative humidity and exposed to 16 hour photoperiod provided by cool fluorescent tubes (3000 lux). Shoot tips and nodal segments were excised from the seedlings, raised on the media, to initiate the primary cultures.

RESULTS

The seeds of *Atriplex hortensis* when inoculated on basal medium resulted in full fledged seedling formation (Fig.1a).

Callogenic response was observed in nodal and shoot tips excised from seedlings when cultured on MS ½ medium supplemented with different concentrations of BAP (Table 1). Pale yellow callus at the base along with the elongation of shoot was observed on MS+ BAP (1.0 µM). Green callus of very high degree was observed on BAP (7.77 µM) (Fig.1b). Pale yellow callus formation at the base of main shoot was observed at MS+ BAP (4.4µM) after six weeks of culture period (Fig.1c). All such calli were non-regenerative type.

More trials in this direction were conducted with two different BAP concentrations with NAA concentrations to see which concentration favours shoot multiplication

Response of *in vitro* raised nodal/shoot tip explants of *Atriplex hortensis* to various BAP and NAA concentrations is given in Table 2.

Table 1. Morphogenetic response of nodal segments and shoot tips of A. hortensis at various concentrations of BAP

INDUCTION MEDIUM	NATURE OF RESPONSE*	DEGREE OF CALLUS FORMATION
MS+BAP (1.0 µM)	Pale yellow Callus at the base	+
MS + BAP(1.11µM)	Pale yellow Callus at the base	+
MA+BAP (4.44µM)	Pale yellow Callus at the base	++
MS+BAP (7.77µM)	Green Callus at the base	+++

Data scored at the end of six weeks of culture period, * Means of 10 replicates

+ low growth, ++ Moderate, +++ high

Table 2: Effect of BAP and NAA on the nodal segments and shoot tips explants of *A. hortensis*

GROWTH MEDIUM	RESPONSE	DEGREE OF CALLUS FORMATION
MS+BAP (7.5μM) + NAA(7.5μM)	Axillary branching and elongation of main shoot	+
MS + BAP (12.5μM) +NAA (12.5μM)	Elongation of main shoot and no axillary branching	+

+low growth

On MS+BAP (7.5μM) + NAA (7.5μM) elongation of shoots with enhanced axillary branching was observed along with low callus formation at the basal end (Fig. 1d). However elongation of shoots without axillary branching was registered on MS+BAP (12.5 μM) + NAA (12.5 μM).

DISCUSSION

Present preliminary findings reveal information regarding morphogenetic potential of nodal segments and shoot tips explants of *Atriplex hortensis*. Enhanced axillary branching was observed by cultivating shoot tips and nodal segments on medium supplemented with combination of 7.5 μM BAP and 7.5 μM NAA. In contrast Uchida *et al.* (2003) have reported that *A gmelini* plants were regenerated via organogenesis from hypocotyl explants. Shoots were regenerated from the callus lines on L.S. medium supplemented with 20 μM TDZ and 0.1μM α -napthalenacetic acid under high – intensity light. However TDZ, which is having cytokinin like activity was, reported to induce not only adventitious and/ or axillary shoot production through organogenesis, but also somatic embryogenesis in apple, wheat, barley and mulberry (Saito and Suzuki 1999; Sugimura *et al.*, 1999; Shan *et al.*, 2000) which is not in line with present studies. The combination of TDZ and NAA at various concentrations was tested to promote regeneration in *Atriplex gmelini* (Uchida *et al.*, 2003). These studies run parallel to our findings as TDZ has cytokinin like activity and with NAA proves effective. Different degrees of callus formation was observed on various concentrations of BAP. However Uchida *et al.*, reported that frequency of callus formation was highest at 1μM BAP and 5μM NAA. Callus proliferation being most prominent on MS medium supplemented with 9.3 *μM* of 6-furfurylaminopurine (kinetin) and 3.39 *μM* 2,4-dichlorophenoxyacetic acid (2,4-D) was reported by Al-Khayri *et al.* (1991) in spinach. In present study callus formation was highest with BAP (7.7 μM).

Present findings indicate that shoot tips and nodal explants of *Atriplex hortensis* possess the potentiality to produce enhanced axillary

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branching which can be used for plantlet formation after following proper rooting procedure in isolated shoots. Micropropagation of *Atriplex* species is very important for the genetic transformation to elucidate the mechanism of salinity tolerance in halophytes.

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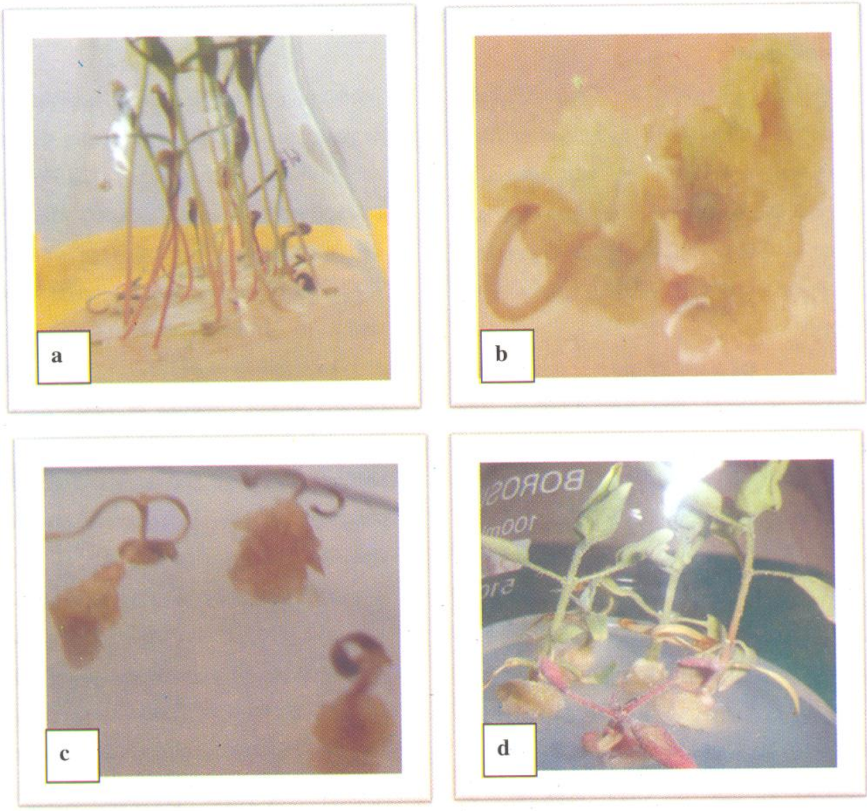

Fig.1 (a-d) ***In vitro*** **culture of *Atriplex hortensis* L.**

(a) *In vitro* seed germination and seedlings formation on MS(1/2) basal medium (after 2 weeks)
(b) Green callus formation in nodal/shoot tip explants on MS+ BAP (7.77 µM) (after six weeks)
(c) Callus formation in nodal/shoot tip explants on MS+ BAP (4.4 µM) (after six weeks)
(d) Axillary branching and elongation of shoots on MS+ BAP (7.5µM)+ NAA (7.5µM) (after 10 weeks.)

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