IN VITRO CALLUS INDUCTION AND SHOOT FORMATION OF *JUNIPERUS EXCELSA* OF ZIARAT, BALOCHISTAN, PAKISTAN

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خلاصہ

اس تحقیق میں صنوبر کے جنگلات کو بڑھانے اور محفوظ کرنے کے لیئے پلانٹ ٹیشو کلچر ٹیکنیک کا استعمال کیا گیا جس میں سات ہارمونز Activated Charcoal (AC), Yeast Extract (YE), L-glutamine (L-G), Polyethylene glycol (PEG), 6-Benzylaminopurine (BAP), 2-4-Dichlophenoxyacetic acid (2,4-D), Indole-3.butyric acid(IBA) دو مختلف ذرائع یا ٹیکنیک (Murashige and Skoogs(MS) and Woody plant (WP) کے تحت استعمال کیے گئے۔ ہر ہامون کی پانچ مختلف مقدار استعمال کی گئیں۔ اِن چار ہارمونز میں Callus جڑ اور تنے کی اچھی طرح افزائش ہوئی تھی، جبکہ PEG1000 کی وجہ سے پودے کی افزائش میں خاطر خواہ تبدیلی نہیں آئی۔ YE اور L-G کی وجہ سے پودے کی افزائش میں ایک مہینے تک بڑھوتری ہوئی لیکن اُس کے بعد پودے کی افزائش رُک گئی اور کوئی پیش رفت نہیں ہوئی۔

Abstract

Effect of Activated Charcoal (AC), Yeast Extract (YE), L-glutamine (L-G), Polyethylene glycol (PEG), 6-Benzylaminopurine (BAP), 2,4-Dichlorphenoxyacetic acid (2,4-D), Indole-3-butyric acid (IBA) was checked to optimize protocol for *in-vitro* growth of *Juniperus excelsa*. The young plant shoots of *J. excelsa* were cultured in Woody Plant (WP) and Murashige and Skoogs (MS) media with five optimal concentration of different phyto-hormones (BAP, 2,4-D, IBA, AC, PEG 1000, YE and L-G). The results showed that BAP, 2,4-D, IBA and AC in both media showed statistically significant regeneration of root and shoot callus. However, PEG 1000 did not affect growth in both media and negatively affected at high concentration. YE and L-G enhanced growth of callus and shoot for 4 weeks in both media after that the callus and shoot formation stopped and no further growth was observed.

Keywords: *juniperus excelsa, Balochistan, in-vitro*

Introduction

Junipers belong to genus Juniperus of the family Cupressaceae (Farjon, 1992). The Genus Juniperus is basically monophyletic (Little, 2006; Adams, 2011) that consists of almost seventy species (EI-Juhany, 2015) spread all over Africa and the Northern Hemisphere (Farjon, 1992). Juniperus is the third largest genus among the conifers found in the world (Andersson and Lhoir, 2006). Plants belonging to this genus grow slowly and can live as long as up to 2000 years. These coniferous plants can be found in all sizes ranging from small flat shrubs to giant forest trees. One thing that stands Junipers out in other plants is that they can survive in different sites enduring extreme and rapid fluctuations in temperature. They can grow in the arid places where other plants cannot survive (El-Juhany, 2015). The economic and environmental importance of Juniper forests make them highly valuable (Daneshvar *et al*., 2014). The coniferous plants make a great source of habitation for wildlife in order to provide shelter against soil erosion (Korouri *et al*., 2012). *J. excelsa* has various medicinal uses for the treatment of different health problems including cough, colds. Tuberculosis, dysmenorrheal and jaundice (Emami *et al*., 2011). The essential oil of *J. excelsa* contains effective medicinal properties in addition to serve as the main ingredient of aromatherapy which is used for scent masks and mood scents. It is also used in making of cosmetics, candles, fragrances, lotions and soaps (Khan *et al*., 2012). Balochistan boasts a large area of juniper forest that are spread in its several isolated and dry valleys (1200m to 3000m above the sea level) (Rafi, 1965). These compacted areas comprise Sasnamana, Sinjawi, and Ziarat forests that cover a huge vicinity of 150, 920 acres. On the other hand, North Zarghun, Babri, Tagha-Tar-Khur, Torshor, and Central Zarghun forests cover a vicinity of 61, 786 acres making it the second largest compact block in Balochistan. *J. excelsa* is the most important specie of Balochistan Juniper forest. The woodlands containing these flowering plants cover a large area of 141,000 hectors including Ziarat and Loralai regions that occupy about 86,000 hectors. Ziarat and Zarghun occupy 100, 000 hectares of area whereas the second-largest forest is found in Herboi hills in Kalat

district (Khatak, 1963). There are several biotic and a-biotic factors that make the Juniper forest of Ziarat exist in a ruined condition (Zaidi *et al*., 2008).

Unfortunately, the Ziarat juniper forest is at a huge risk of devastation because of over-grazing, excessive cutting and over-exploitation by the rural population for house hold and medicinal purposes. Zaidi *et al*., (2012) reported that Micro-propagation is an effective technique and also optimized different protocols of Junipers. This paper optimized the protocols for *in-vitro* micro-propagation of *J. excelsa* for its conservation, afforestation and restoration of Juniper forest of Ziarat and different phyto-hormones.

Materials and Methods

Collection and Preparation of Plant Material

The young shoots of *J. excelsa* were collected from Ziarat Forest Balochistan. The young plant shoots were used for micro-propagation these were inoculated on different media. The explants were washed under the tap water.

Preparation of Media

Preparation of Stock Solutions

MS basal medium (Murashige and Skoogs, 1962) and Woody Plant medium, Lloyd and McCown, 1980) all the supplements with different growth hormones were tested for the callus induction and for the shoot formation. In the culture medium stock solutions of nutrients used for the sake of accommodation and precision. All the stock solutions were prepared by using analytical grade chemicals and double distilled water.

Growth Regulators

Stock solutions of growth regulators were either prepared in mM or µM concentrations and were used according to the requirement of the medium. The plant growth regulators used in this research were 6-Benzylaminopurine (BAP), 2,4-Dichlorphenoxyacetic acid (2,4-D), Indole-3-butyric acid (IBA), Polyethylene glycol (PEG), Activated Charcoal (AC), Yeast Extract (YE) and L-glutamine (L-G).

Preparation and Sterilization of Explant

First the explants were washed in tap water and then treated with the plant detergent for 10 minutes. Then the explants were transferred in distilled water to remove the traces of the detergent and then treated with 2.5% of chlorine from sodium hypochlorite for 15 minutes. Explants were washed with sterilized or autoclaved distilled water for three or four times and divided the explants or the shoots tips 1-1.5 cm in length. The explants were cultured in MS and WP media.

Result and Discussion

The meristematic tissues of young shoot tips were used as explant for callus induction. The free pathogens explants were cultured on two types of medium at five different concentrations of phyto-hormones (0, 0.25, 0.50, 0.75, 1.0 mg/L). Media such as WP and MS were used to optimize the protocol for the growth. To optimize the media for regeneration and proliferation of callus shoots and roots formation different concentration of growth hormones were used (6-Benzylaminopurine (BAP), 2,4-Dichlorphenoxyacetic acid (2,4-D), Indole-3-butyric acid (IBA), Polyethylene glycol (PEG), Activated Charcoal (AC), Yeast Extract (YE) and L-glutamine (L-G)). Data presented in Fig. 1-12 show effects of different combinations of phyto-hormones supplemented in WP and MS Media. Increase or decrease in the high concentration of hormone adversely affected the rate of callus, shoot, root formation and growth.

Fig.1, Showed that the highest growth of callus and shoot was observed at 0.50(mg/L) concentration of BAP/2,4-D in WP media after 9 weeks. Almost similar results were found to Zaidi *et al*., (2012) they reported that the maximum growth of callus and shoots were recorded at 0.5 (mg/L) concentration of BAP/2, 4-D in WP media within 8 weeks. while the MS media 0.75(mg/L) concentration the callus and shoots were found within 10 weeks (Fig. 2). 50% growth was seen at AC 0.75(mg/L) in WP media while in MS media it showed 40% growth at AC 0.50(mg/L) (Fig. 4 and 3). Similarly, to Wang *et al*., (1976) reported that only at 3g per liter of AC obtained maximum shoot tips of ginger. Normah *et al*., (1995) also reported that at $2gl^{-1}$ AC produced little shoots of *Garcinia mangostana* L in MS media.Figure 5 and 6 showed the response of IBA in WP/MS media for root formation. The best roots were observed at IBA 0.1 (mg/L) concentrations in WP media in 6 weeks but in MS media at IBA 0.75(mg/L) concentration maximum roots were found in 6 weeks. Zaidi *et al*., (2012) also reported similar results that the maximum roots formation was observed at IBA 0.1 (mg/L) concentrations in WP media in 6 weeks. However, first time PEG (1000 molecular weight) was used to check its effect on *J. excelsa* growth.

Effect of growth regulators on callus and shoot formation of *Juniperus excelsa* using MS and WP media:

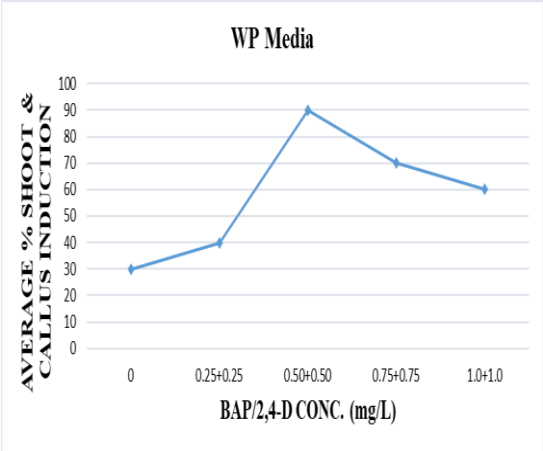

Fig. 1. Bar chart representing the response of BAP and 2, 4-D in WP medium

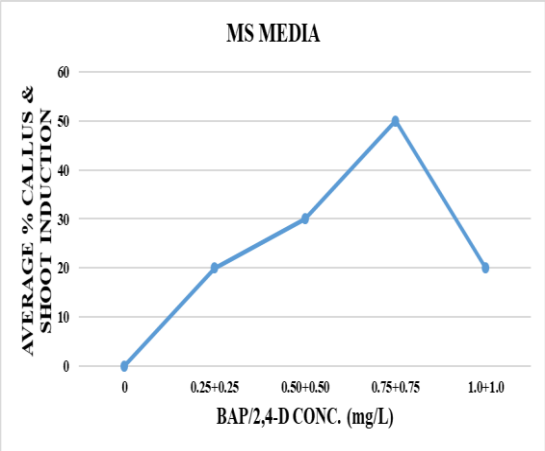

Fig. 2. Bar chart representing the response of BAP and 2, 4-D in MS medium.

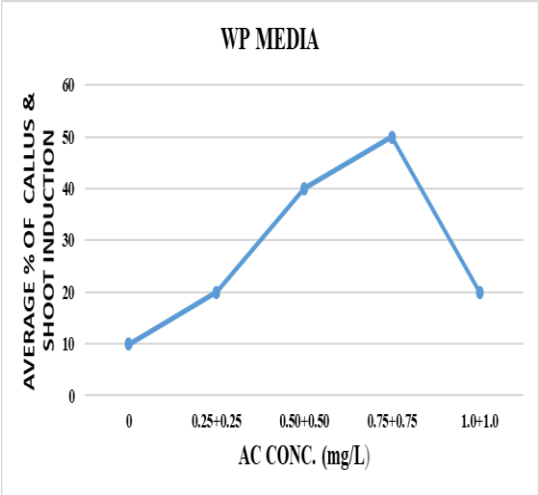

Fig. 3. Bar chart represented the response of Activated Charcoal in WP medium

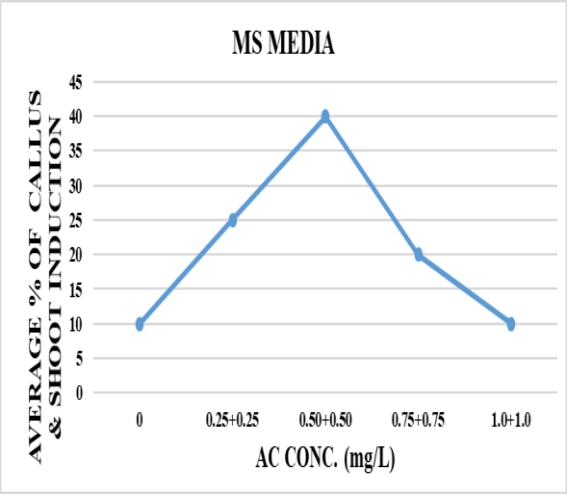

Fig. 4. Bar chart represented the response of Activated Charcoal in MS

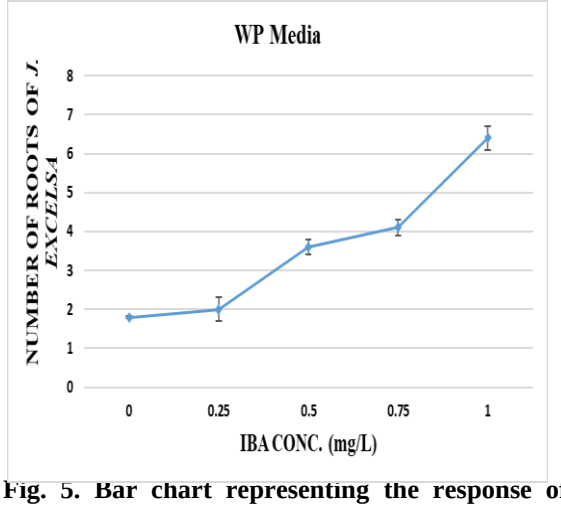

Fig. 5. Bar chart representing the response of IBA in WP medium

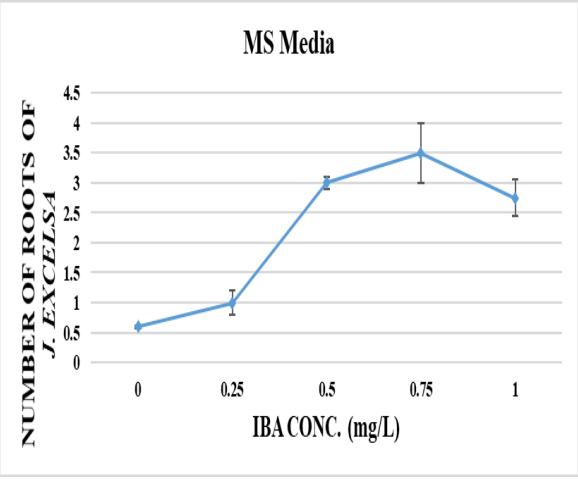

Fig. 6. Bar chart representing the response of IBA in MS

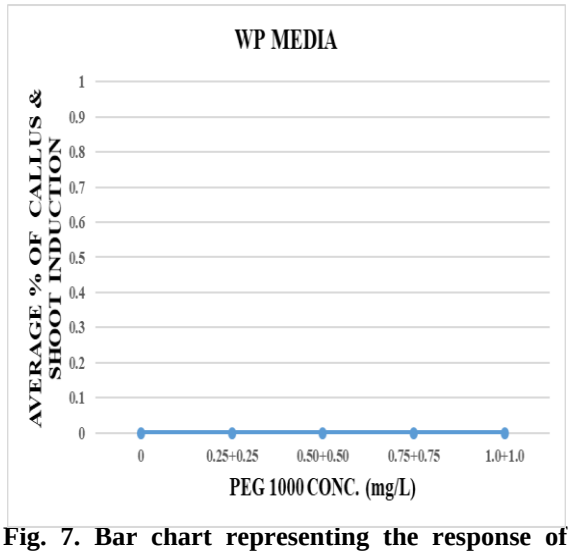

Fig. 7. Bar chart representing the response of PEG 1000 in WP medium.

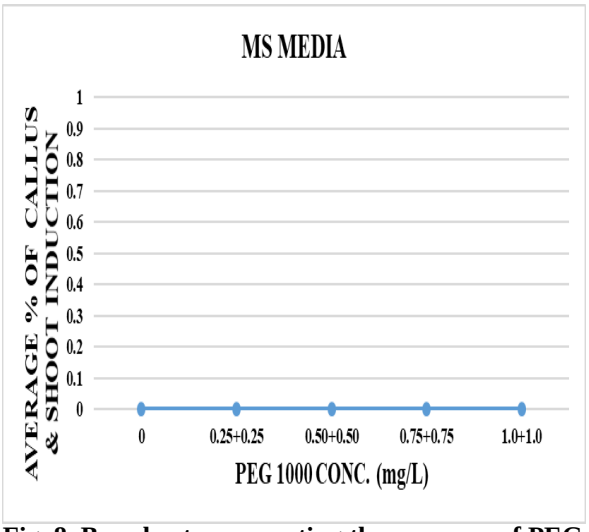

Fig. 8. Bar chart representing the response of PEG 1000 in MS medium

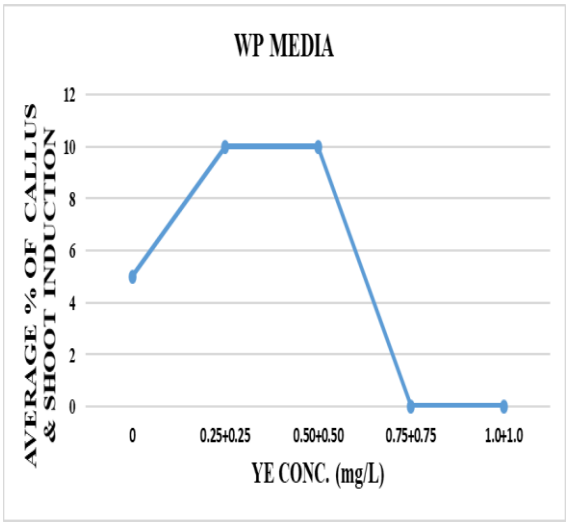

Fig. 9. Bar chart representing the response of Yeast Extract in WP medium.

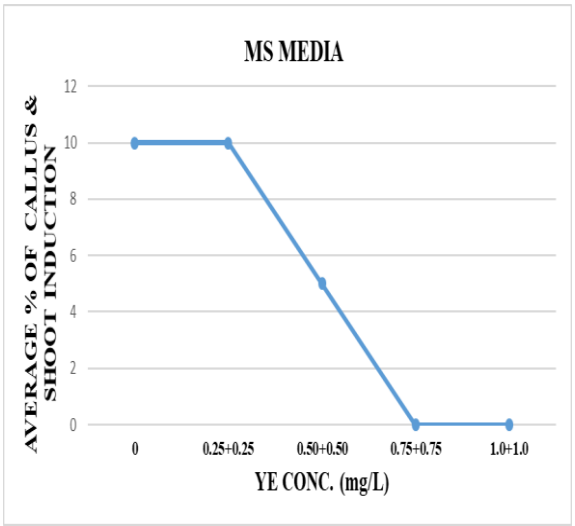

Fig. 10. Bar chart representing the response of Yeast Extract in MS medium

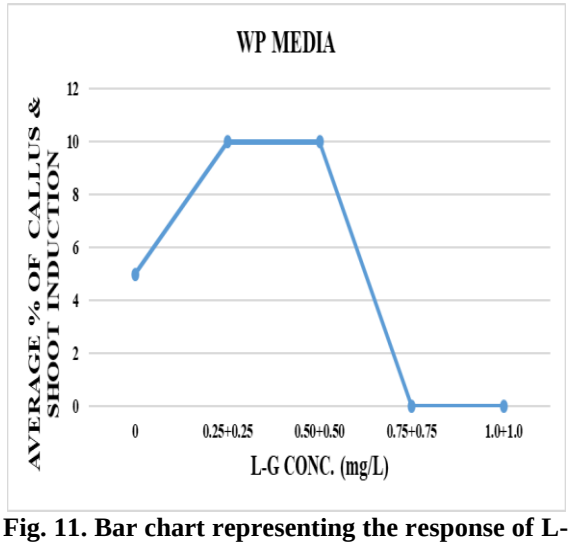

Fig. 11. Bar chart representing the response of L-Glutamine in WP medium.

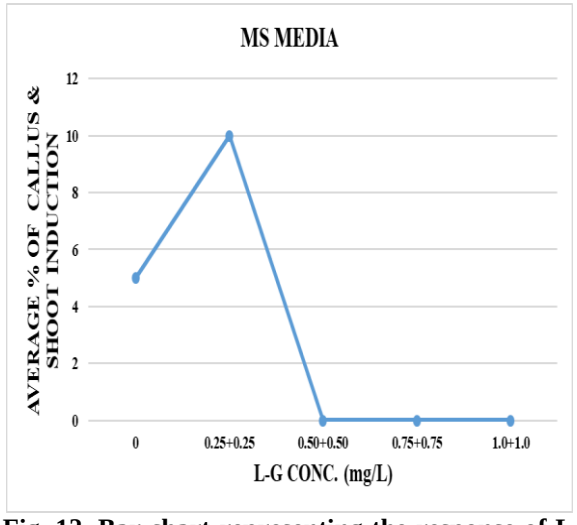

Fig. 12. Bar chart representing the response of L-Glutamine in MS medium.

After many trials in all concentrations of PEG (1000 molecular weight) no positive growth was seen, there was no growth in both MS and WP media (Fig. 7-8). Also in Maize PEG (3,350 molecular weight) did not showed any improvement in plant regeneration ability Abdel-Rahman and Widholm, (2010). Yeast extract and L- glutamine were first time used for regeneration of *J. excelsa* in WP and MS media. Fig. 9-12 showed that in beginning from 4 weeks the shoots and callus regeneration was seen but after that the while explants became black which means no growth had shown but Robbins, (1922) reported that yeast extract were effective at the root tips of tomato *in-vitro* also Abraham *et al*., (2010) presented that yeast extract did not show significant result on the shoot formation of *in-vitro Curcuma manga*. Shahsavari, (2011) presented that in upland rice glutamine had shown significant growth but in pineapple L-glutamine showed low in shoot-bud induction (Hamasaki *et al*., 2005). Regeneration of shoots and roots from tissue and cell culture can be actuated by increasing and decreasing the phyto-hormones ratio in the culture medium. Since different species have responded to the treatment with exogenous phyto-hormones. The optimized phyto-hormones ratio that differentially catalyze maximum level of morphogenesis is imperative for proficient of micropropagation.

Conclusion

This study present and established suitable media with different composition of phyto-hormones for *in-vitro* growth of *J. excels*. Concluded that the highest growth of callus and shoot was observed at 0.50(mg/L) concentration of BAP/2,4-D in WP media after 8 weeks, while in MS media at 0.75(mg/L) concentration of BAP/2,4-D showed maximum growth of callus and shoot. Maximum roots formation was observed at IBA 0.1 (mg/L) concentrations in WP media in 6 weeks but in MS media at IBA 0.75(mg/L) concentration maximum roots were found in 6 weeks. The WP media is best for *in-vitro* propagation of *J. excelsa*. Activated Charcoal was first time used in micropropagation of *J. excelsa* which was found effective. When treated with PEG 1000 in MS and WP media which did not work and showed negative effect all the cultures turned brown to black in one-week time. Yeast extract and L-glutamine also did not show any effective regeneration but was also not harmful and caused death of the cell culture and no growth was observed.

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References

Abdel-Rahman, M. M., and Widholm, J. M. (2010). Maize tissue culture plant regeneration ability can be improved by polyethylene glycol treatment. *In Vitro Cellular and Developmental Biology-Plant*, *46*(6), 509-515.

Abraham, F., Bhatt, A., Keng, C. L., Indrayanto, G., and Sulaiman, S. F. (2011). Effect of yeast extract and chitosan on shoot proliferation, morphology and antioxidant activity of Curcuma mangga in vitro plantlets. *African Journal of Biotechnology*, *10*(40), 7787-7795.

Adams, R. P. (2011). The junipers of the world: The genus Juniperus.–Victoria. British Columbia: Trafford Publishing.

Andersson, F. and P. Lhoir. 2006. Introduction. Conifers. Ecosystems of the World 6. Conifer Forests. 1-22.

Daneshvar, A., Tigabu, M., Karimidoost, A., Farhadi, M., and Odén, P. C. (2014). Growth characteristics and reproductive output of dwarf mistletoe-infected *Juniperus polycarpos* in Iran. *Journal of forestry research*, *25*(4), 827-834.

El-Juhany, L. (2015). The Magnitude of Dieback on Juniperus procera Trees in the Natural Forests in the Southwestern Region of Saudi Arabia. Biosciences, *Biotechnology Research Asia, 12*(1), 219-230.

Emami, S. A., Abedindo, B. F., and Hassanzadeh-Khayyat, M. (2011). Antioxidant activity of the essential oils of different parts of Juniperus excelsa M. Bieb. subsp. excelsa and J. excelsa M. Bieb. subsp. polycarpos (K. Koch) Takhtajan (Cupressaceae). Iranian journal of pharmaceutical research: *IJPR*, *10*(4), 799.

Farjon, A. (1992). The taxonomy of multiseed Junipers (Juniperus Sect. Sabina) in southwest Asia and east Africa (Taxonomic notes on Cupressaceae I). Edinburgh Journal of Botany. 49: 251-283.

Hamasaki, R. M., Purgatto, E., and Mercier, H. (2005). Glutamine enhances competence for organogenesis in pineapple leaves cultivated in vitro. *Brazilian Journal of Plant Physiology*, *17*(4), 383-389.

Khan, M., Khan, A. U., and Gilani, A. H. (2012). Pharmacological explanation for the medicinal use of Juniperus excelsa in hyperactive gastrointestinal and respiratory disorders. *Journal of natural medicines*, *66*(2), 292-301.

Khattak, G.M. (1963). Working plan for Juniper forests of Quetta civil division. Govt, Printing Press, West Pakistan.

Korouri, S. A. A., Khoushnevis, M., and Matinizadeh, M. (2012). Comprehensive studies of Juniperus species in Iran. Forest, Range and Watershed Management Organization (In Persian with English Abstract).

Little, D. P. (2006). Evolution and circumscription of the true cypresses (Cupressaceae: Cupressus). *Systematic Botany*, *31*(3), 461-480.

Lloyd, G. and B. McCown. (1980). WPM Commercially feasible micropropagation of mountain laurel, Kalmia latifolia, by use of shoot tip culture. *Proc. Int. Comb Plant Prop Soc.*, *30*, 421-427.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia plantarum*, *15*(3), 473-497.

Normah, M. N., Nor-Azza, A. B., and Aliudin, R. (1995). Factors affecting in vitro shoot proliferation and ex vitro establishment of mangosteen. *Plant cell, tissue and organ culture*, *43*(3), 291-294.

Rafi, M. (1965). Vegetation types of Balochistan Province. Pakistan Govt, Printing Press, Lahore, Pakistan.

Robbins, W. J. (1922). Cultivation of excised root tips and stems tips under sterile conditions. *Botanical gazette*, *73*(5), 376-390.

Shahsavari, E. (2011). Impact of tryptophan and glutamine on the tissue culture of upland rice. *Plant Soil Environ*, *57*(1), 7-10.

Wang, P. J., and Huang, L. C. (1976). Beneficial effects of activated charcoal on plant tissue and organ cultures. *In Vitro Cellular and Developmental Biology-Plant*, *12*(3), 260-262.

Zaidi, M.A, S. Khan, N. Janan, A, Yousafzai and A. Mansoor. (2012). Micropropagation and conservation of three Juniperus species (Cupressaceae). *Pak. J. Bot.*, *44*, 301-304.

Zaidi, M.A., A. Huda and S.A Crow, Jr. (2008). Biological activity and elemental composition of *Arceuthobium oxycedri* (Dwarf mistletoe) of Juniper forest of Ziarat, Pakistan. *Acta Botanica Hungarica.*, *50*(1-2), 223-230.