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CALLOGENESIS POTENTIAL OF COTYLEDONARY EXPLANTS OF *ALTHAEA ROSEA* L. FROM PAKISTAN

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Abstract

The purpose of this study was to optimize conditions for callogenesis and organogenesis of *Althea rosea* using cotyledonary explants. Varying concentrations solo and in combinations of auxins and cytokinins listing IAA, NAA, 2, 4 D, IBA, BAP and Kinetin were experimented. Among solo auxins, maximum Callus Index (CI= 560) was obtained at 0.03mg/L 2, 4-D after 10 days of initiation. Among cytokinins, 8 mg/L and 10mg/L BAP exhibited good callogenic response (CI= 320). Combinations of BAP+ NAA 4:3.0mg/L and BAP + 2, 4 D 4:0.03 mg/L were found better (CI= 480). Morphological characters of calli were also observed including health, color and texture. This protocol can be exploited to fulfill ornamental, medicinal and industrial demand of *A. rosea* through the latest technique of tissue culture.

Introduction

Althaea rosea (garden Hollyhock, locally known as Gulekhera) is an ornamental and medicinal plant species belonging to mallow family (*Malvaceae*). It is cultivated for variety of colors including white, pink, and red. It is native to China. The plant grows best in medium-fertile, moist, but well-drained soil (Still, 1994) and Clay soil (Abraham, 1999). It has great potential to tolerate and accumulate high concentrations of cadmium (Liu *et al*., 2009; Liu *et al*., 2008). In addition, Holly hock is reported for manufacturing paper used for wrapping and making paper bags (Matsumae *et al*., 1956).

Family Malvaceae appreciated much for *Gossypium* spp., (cotton) and *Abelmoschus esculentus* (okra). Plant tissue culture plays role in crop improvement and propagation (Memon *et al*., 2010; Khan and Ahmad, 2011) Embryogenesis and organogenesis in cotton has been extensively studied (Memon *et al*., 2010; Efe, 2005; Stewart & Hsu, 1977) but there is sporadic information on tissue culture response of *Althea rosea.* Various research groups throughout world have carried out studies on tissue culture of different plant species from Malvaceae (Sie *et al.,* 2010; Raoul *et al.,* 2010; Ruan, *et al*., 2009; Ozyigit *et al*., 2007; Ouma *et al*., 2004; Zapata *et al.,* 1999; Mangat & Roy, 1986; Gunay & Rao, 1978) but protocol for *Althea rosea* tissue culture has not been developed. Although most of tissue culture work in *Malvaceae* is on cotton because of its economic importance (Kishor *et al*., 1982; Morozora & Sinevich 1983; Lev *et al*., 1985; Gooding & Berlin, 1985) some other species like *Sida*, *Abutilon* (Nataraja & Patil 1980) *Malva* (Dexiang *et al*., 1986) and *Hibiscus* (Mizukami *et al*., 1988 and Yutako *et al*., 1989) has also being exploited through tissue culture techniques.

This plant family is appreciated much for number of natural dyes extracted from its petals (Ferenczi *et al*., 1981; Rakhimkhanov *et al*., 1983; Srivastava & Jain 1984; Kasumov, 1984; Salikhov & Idriskhodzhaev, 1978; Otakulov *et al*., 1989). *A. rosea* has been reported to contain highest amount of tannins (Salikhov & Khabibov, 1979; Jozef *et al*., 1983, carbohydrates (Karawya & Afifi, 1979), cyanides (Afifi & Karawya, 1988; Tamas & Stoleriu, 1976) and *Althaea* mucilage (Tomoda *et al*., 1983). Purple red pigment has been extracted from the petals of *Althaea rosea* directly (Jian *et al.,* 2005) and with the help of macroporous resin (Li-song & Yin-hai, 2009).

This plant is reported as emmenagogue (Dudek *et al*., 2006), expectorant used in Unani medicine (Shome *et al*., 1992). Flavonoids isolated from *A. rosea* are used as raw material for pharmaceutical preparations (Matlawska, 1992). Anthocyanin extracted from this plant species are used for obtaining natural drugs having anti-inflammatory, anti-microbial effects, influence on gastrointestinal tract (Iauk *et al*., 2003; Ciculei *et al*., 1990; Takeda *et al*., 1989). The production of copious callus can ensure continuous supply of the plant product for pharmaceutical and food industry that will enhance rapid production of economically important plants like *Althea rosea*.

Materials and Methods

The whole research work was conducted in biotechnology laboratory, Government College University, Lahore. All the experiments were conducted in triplicates.

Preparation of medium: MS medium (Murashige & Skoog, 1962) with varying concentrations of auxins and cytokinins solo and in combinations was used. Medium was supplemented with 3% (w/v) sucrose and 0.7 % agar. The pH of the medium was adjusted at 5.7 and autoclaved under 15 lbs/inch^2 pressures for 15 minutes. The medium was poured in sterilized culture vessels.

Surface sterilization of seed: All the seeds were surface sterilized in 0.01% $HgCI_2$ solution. The explants were properly rinsed with double distilled autoclaved water to remove traces of $HgCl_2$.

Preparation of explants: Seeds were germinated aseptically to obtain explants. Four days old seedlings were used for the procurement of explants. Cotyledons were excised (5mm long) under aseptic conditions. All the explants were inoculated on to media for callogenesis. Callus index (CI) for quantitative estimation of calli initiated and grown under chemical variability was calculated following Khosh & Sing (1982).

Results and Discussions

Effects of plant growth regulators (PGRs) including IAA, NAA, 2, 4 D, IBA, BAP and Kinetin was investigated for callogenesis. Different concentrations viz., 2, 4, 6, 8 and 10mg/L were used for IAA, NAA, BAP and kinetin, whereas in the case of 2,4 D, the concentrations were ranged at 0.01-0.06mg/L. Combinations of IBA+NAA, BAP+NAA were ranged from 4:2.0- 4:4.0; while BAP+ 2,4 D, the same was ranged from 4:0.01-4:0.05 respectively (Table 1). In the case of solo application of PGRs IAA, 2,4 D and Kinetin, compact yellow callus was produced. The BAP also produced yellow callus but it was granular nature, whereas NAA produced loose white callus. Minimum time taken for the initiation of callus was 25 days for BAP, 19 days for IAA, NAA, Kinetin, whereas 2,4D took only 9 days for the same. Three sets of combinations of PGRs were used each represented by five combinations. IBA + NAA combination, callus texture was compact in four and loose callus in one combination. Color of callus was yellow in two cases, green in two and white in only one. BAP + NAA combination callus texture was granular in three and loose in one combination, whereas color of the callus was yellow in two, green in two and white in one. BAP + 2,4D combination callus texture was compact in three, loose in one and granular in one combination, whereas color of the callus was white in four and yellow in one.

MS medium supplemented with IAA (6mg/L) (Fig. 1) gave a Callus Index of 400 in 19 days, whereas at a concentration of 10 mg/L, Callus Index was 80 after 28 days. The NAA 4mg/L exhibited 320 Callus Index after 19 days, whereas, at 2 mg/L, Callus Index was produced 80 in 28 days (Fig. 2). Application of 2, 4 D at the strength of 0.03mg/L produced a Callus Index of 560 after 10 days. In the case of 0.05 mg/L and 0.06 mg/L of 2, 4 D produced a Callus Index of 160 after 14 and 15 days respectively (Fig. 3). Employment of BAP at 8mg/L and 10 mg/L gave rise to a Callus Index of 320 each after 25 and 28 days respectively (Fig. 4). The Kinetin at 4-8 mg/L gave forth a Callus Index of 160 after 26, 24 and 19 days, while 2 mg/L Kinetin induced a Callus Index of 80 after 29 days (Fig. 5).

Table 1. Effects of different concentrations of auxins and cytokinins on cotyledons.

Hormone	Concentration mg/L	Initiation of callus (Days)	Texture of callus	Callus color
IAA	2	20	Compact	Yellow white
	4	23	Compact	Yellow white
	6	19	Compact	Yellow white
	8	25	Compact	Yellow white
	10	28	Compact	Yellow white
NAA	2	28	Loose	White
	4	19	Loose	White
	6	22	Loose	Yellow
	8	25	Loose	Yellow green
2,4 D	0.01	13	Compact	Yellow
	0.02	12	Compact	Green
	0.03	10	Compact	Yellow green
	0.04	9	Compact	Yellow green
	0.05	14	Compact	Yellow green
	0.06	15	Compact	Yellow green
BAP	2	39	Granular	Yellow
	4	38	Granular	Yellow
	6	36	Granular	Light green
	8	25	Granular	Light green
	10	28	Granular	White
Kinetin	2	29	Compact	White
	4	26	Compact	Yellow
	6	24	Compact	Yellow green
	8	19	Compact	Yellow
IBA + NAA	4:2.0	22	Compact	White
	4:2.5	20	Compact	Green
	4:3.0	15	Loose	White
	4:3.5	13	Compact	Yellow
	4:4.0	10	Compact	Yellow
BAP + NAA	4:2.0	13	Granular	Green
	4:2.5	12	Compact	Green
	4:3.0	10	Loose	Yellow
	4:3.5	16	Granular	Yellow
	4:4.0	20	Granular	White
BAP + 2,4D	4:0.01	15	Compact	Yellow
	4:0.02	13	Loose	White Green
	4:0.03	12	Granular	White
	4:0.04	14	Compact	White
	4:0.05	16	Compact	White

Combinations of auxins and cytokinins were also tested for callus induction. Employment of IBA + NAA at 4:4.0 mg/L produced a Callus Index of 320 after 10 days of incubation (Fig. 6). In the case of NAA+ BAP at the ratio of 4: 3.0 mg/L resulted in Callus Induction of 480 in 10 days (Fig. 7). The combined action of BAP+ 2, 4 D at the ratio of 4:0.03mg/L developed 480 Callus Induction after 12 days (Fig. 8). Maximum Callus Index (560) was observed at 0.03mg/L 2, 4 D after 10 days, while minimum Callus Index (80) was recorded in each of 10mg/L IAA, 2 mg/L NAA, 2 mg/L BAP, 2 mg/L Kinetin and 4.0: 2.0 mg/L, 4.0: 2.5 mg/L IBA+NAA, 4:4.0mg/L BAP+NAA and 4:0.01 mg/L, 4:0.02 mg/L BAP + 2,4 D after 28, 28, 39, 29, 22, 20, 20, 15 and 13 days respectively. The best hormone for Callus induction of *Althea rosea* was found to be 2, 4 D at a concentration of 0.03 mg/L in this study. Similar result was shown by Efe (2005) on *Gossypium* spp. using 3 days old cotton ovules as explant source by employing different combinations of auxins and cytokinins for callogenesis, but the growth regulator employed was IAA+Kinetin combinations as opponent to our work in which IAA+NAA, BAP+NAA and BAP + 2,4 D combinations were experimented.

In another study, Mushtaq *et al*., (1994) investigated callogenesis of *A. rosea* nodal explants. They reported that 8 mg/L BAP and 0.8 mg/L 2 ip were equally effective in callus induction as opposed to our study in which 2, 4 D was found to be the best for callogenesis. Among various explants that have been used so far for callogenesis and organogenesis, cotyledons have been reported by Hamidou *et al.*, 2001, Iriondo & Perez, 1992, Triplett *et al.*, 2008 and Sie, 2010.

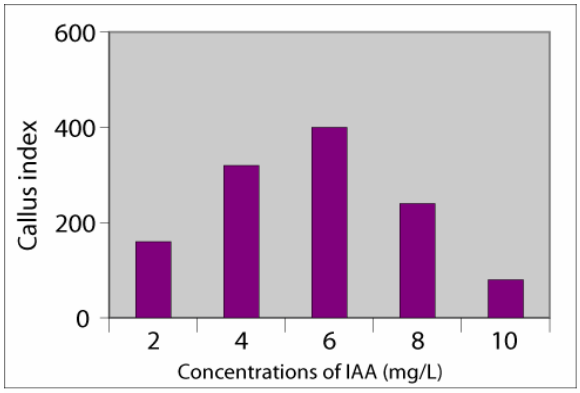

Fig. 1. Effects of IAA on callus induction.

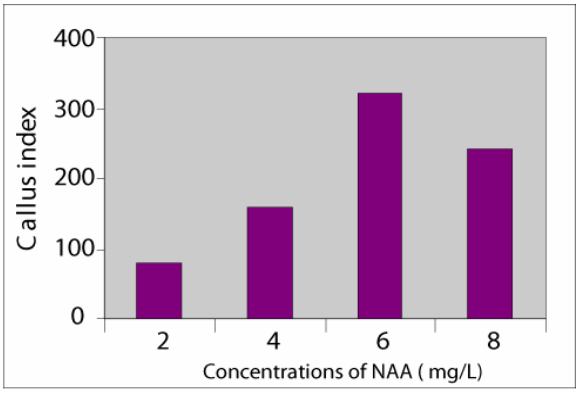

Fig. 2. Effects of NAA on callus index.

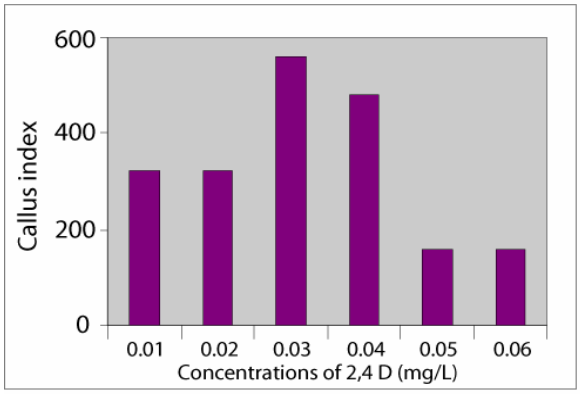

Fig. 3. Application of 2, 4 D for callus induction.

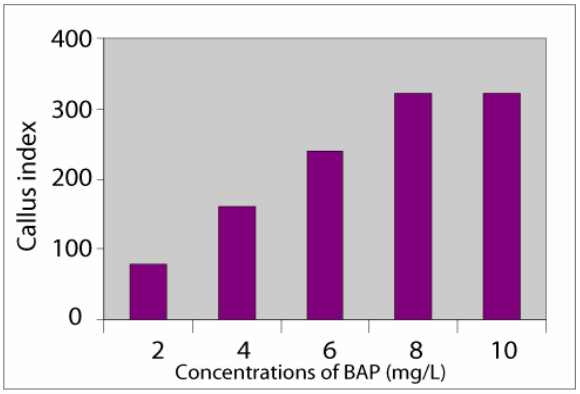

Fig. 4. Different concentrations of BAP for callus induction.

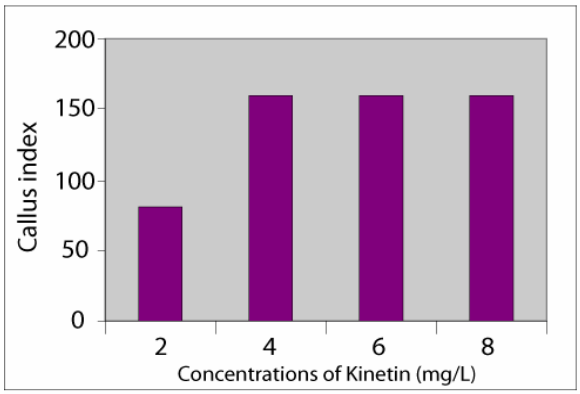

Fig. 5. Callus Index by Kinetin.

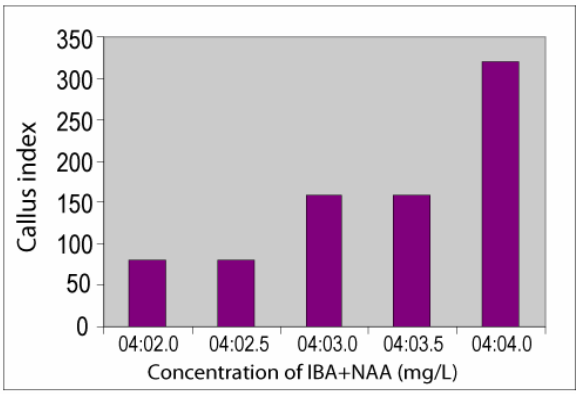

Fig. 6. Combination of IBA+NAA for callus induction.

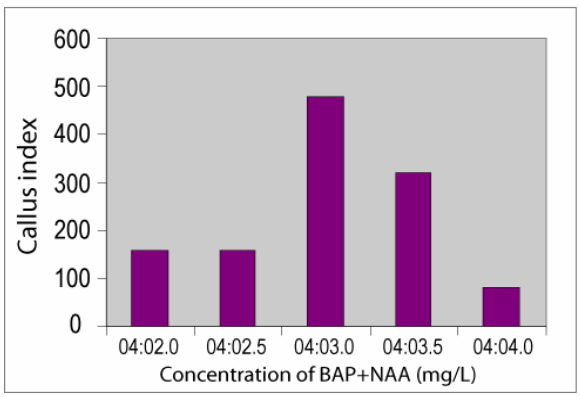

Fig. 7. Combination of BAP+NAA for callus induction.

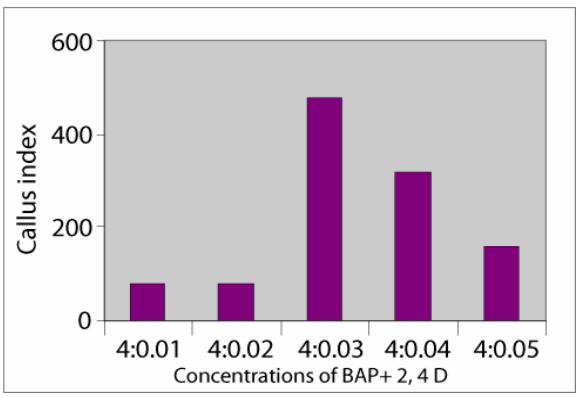

Fig. 8. Combination of BAP+2, 4D for callus induction.

References

Abraham, D.K. 1999. *The Green Thumb Garden Handbook*. Lyons Press, 528 pp.

Afifi, M.S and M.S. Karawya. 1988. Investigation of flower anthocyanin pigments of *A. rosea* Cav. and, *Malva viscous-arborea* L. growing in Egypt. *Bull. Pharm. Sci.*, 11(2): 185-95.

Ciculei, I., V. Istudor, M. Palade, D. Albulescu and E. Crista. 1990. Phytochemical and pharmacological research on anthocyanins for obtaining new natural drugs. *An.R. Acad. Farm*. 56(4): 351-7.

Dexiang, N., C. Ghang, Z. Pifang and W. Kaiji. 1986. Effect of wavelength on peroxidase isoenzymes and root formation in the callus cultures of *M. sylvestris*, Fudan xuebae, Niran kexueban. Journal Name 25(2): 157-62.

Dudek, M., M. Irena and S. Maurycy. 2006. Phenolic acids in the flowers of *A. rosea* Var. nigra. *Acta Poloniae Pharmaceutica- Drug research*, 63(3): 207-211.

Efe, L. 2005. Callus formation and plant regeneration from two Cotton species (*Gossypium hirsutum* L., and *G. B*arbadense L.). *Pak. J. Bot*., 37(2): 227-236.

Ferenczi, S., M. Kallay and G.H. Bardi. 1981. Natural dyes preparations from Hollyhock petals. Teljes HU. 26: 789.

Gooding, J.R. and J.D. Berlin. 1985. *In vitro* production of cotton fibers. *Eur. plant. Appl. Ep*, 882.

Gunay, A.L. and P.S. Rao. 1978. *In vitro* Plant regeneration from hypocotyls and cotyledon explants of red pepper (*Capsicum*). *Plant Science Letters*, 11(3-4): 365-372.

Hamidou, S.F., Z. Allan, R. Kanniah, S. Sukumar and S.C. Govind. 2001. Induction of highly embryogenic calli and plant regeneration in upland cotton (*Gossypium hirsutum* L.) and pima (*Gossypium barbadense* L.). *Crop Sci.*, 41: 1235-1240.

Iauk, L., A.M.L. Bue, I. Milazzo, A. Rapisarda and G. Blandino. 2003. Antibacterial activity of medicinal plant extracts against periodontopathic bacteria. *Phytotherapy Res*, 17(6): 599-604.

Iriondo, J.M. and C. Perez. 1992. *In vitro* plant regeneration of *Lavatera oblongifolia* (Malvaceae), an endangered species Botanic Garden Micropropagation News., 1(5): 54-57.

Jian, S., P. Zi-mo and J. Yue-ming. 2005. Studies on the Purple-red Pigments from Flowers of *Althaea rosea* (L.) Cavan. *Food Science, China*, 26(10): 66-71.

Jozef, M., M.S. Zsuzsanna, P.K. Nadori, M. Hager-veres and A. Hung. 1983. Refreshing and an invigorating beverage concentrate. *J. Pharm. Sci*., 28(1): 539-542.

Karawya, M.S. and M.S. Afifi. 1979. Investigation of carbohydrates of Egyptian *A. rosea* and *Malve arborea* L. Egypt. *J. Pharm. Sci*., (pub.1982). 20(1-4): 291-8.

Kasumov, M.A. 1984. *Red dye from the holly hock and its use in food industry*. Doklady-Akademiya Nauk Azerbaidzhanskoi, SSR, 40(6): 76-79.

Khan, M.A. and J. Ahmad. 2011. *In vitro* wheat haploid embryo production by wheat x maize cross system under different environmental conditions. *Pak. J. Agri. Sci*., 48: 39-43.

Khosh, M. and K. Sing. 1982. Callus induction and culture of roses. *Sci.Hortic*.17: 361.

Kishor, P., B. Kavi and A.R. Mehta. 1982. Some aspects of carbohydrates metabolism in organ-differentiating tobacco and non-differentiating cotton callus cultures. Proc. Int. Congr.plant Tissue cell culture 5th Edition, pp.143-45.

Lev, S.V., G.A. Khodzhaeva and D.A. Museav. 1985 .Growth regulators for tissue culture of some *Gossypium* spp. Doki.Akad.Nauk.UZ SSR, (6): 52-56.

Li-song, X.U and M.A. Yin-hai. 2009. Use of Macroporous Resin for the Extraction of Red Pigments from *Hibiscus sabdariffa* L. *Food Science*, 12-23.

Liu, J.N., Q.X. Zhoub, T. Sun, L.Q. Mad and S. Wang. 2008. Growth responses of three ornamental plants to Cd and Cd–Pb stress and their metal accumulation characteristics. *J. Hazardous Materials*, 151(2008): 261-267.

Liu, J.N., Q.X. Zhou, S. Wang and T. Sun. 2009. Cadmium tolerance and accumulation of *A. rosea* Cav. and its potential as a hyperaccumulator under chemical enhancement. *Environ. Monit. Assess.*, 149(1-4): 419-27.

Mangat, B.S. and M.K. Roy. 1986. Tissue Culture and plant regeneration of Okara (*Abelmoshous esculentus*). *Plant Sciences*, 47(1): 57-61.

Matlawska, I. 1992. Flavonoids in the flowers of *A. rosea* Var. niger (Malvaceae). *Herba Polon*, 38(4): 163-72.

Matsumae, R., S. Rani and K. Motoki. 1956. Preservation test of holly hock yam extract by xylon. *Echime Ken. Sei Shi, Shik. Hoko*, 9: 9-13.

Memon, N., M. Qasim, M.J. Jaskani and R. Ahmad. 2010. *In vitro* cormel production of gladiolus. *Pak. J. Agri. Sci*., 47: 115-123.

Memon, S., S.N. Mari, A.K. Mari and N.H. Gaddi. 2010. Induction of callus through anther and ovule culture in upland Cotton (Gossypium hirsutum L.). *World Applied Sciences*, (*Special Issue of Biotechnology & Genetic Engineering*), 8: 76-79.

Mizukami, H., K. Tmita, H. Ohashi and N. Hiraoka. 1988. Anthocyanin production in callus cultures of *Hibiscus sabdariffa*. *Plant Cell Rep*., 7(7): 553-556.

Morozora, A.V. and L.I. Sinevich. 1983. Growth of cotton fibers and ovules under different culture conditions. *Dokl.Vses.Akad. S-kh.Naukim*. 1(9): 20-22.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and-bio assays with tobacco tissue cultures. *J. Physiol*., 15: 473-497.

Mushtaq, H., A. Hussain, M. Akram and F.H. Shah. 1994. Growth conditions for callogenesis in *Althaea rosea* (L.) Cav. *Pak. J. Agric. Res.*, 15(1): 37-42.

Nataraja, K. and J.S. Patil. 1980. Effect of auxins and Kinetin on *in vitro* regeneration in tissue cultures of *Abutilon indicum* Sweet. *Ind. J. Exp. Biol.*, 18(4): 433-35.

Otakulov, M.K., S.A. Salikhov and T.T. Saidova. 1989. Hollyhock pigments. (Tash kent. Inst. Nar. Khoz., Tashkent, USSR). *Pishch. Prom*., (9): 43-44.

Ouma, J.P., M.M. Young and N.A. Reichert. 2004. Optimization of *in vitro* regeneration of multiple shoots from hypocotyls sections of cotton (*Gossypium hirsutum L.*). *African J. Biotech.*, 3(3): 169-173.

Ozyigit, I.I., M.V. Kahraman and O. Ercan. 2007. Relation between explant age, total phenols and regeneration response in tissue cultured cotton (*Gossypium hirsutum* L.). *African J. Biotech.*, 6(1): 003-008.

Rakhimkhanov, Z.B., A.K. Karimdzhanov, A.I. Ismailov and F.K.H. Mukhamedova. 1983. Red food colouring from plants. *(Inst. Bioorg. Khim, USSR). Uzb. Khim.Zh*., (5): 46-9.

Raoul, S.S., C. Gilbert, S.F. Hamidou, T. Yannick, D. Yao, S. Abdourahamane and B. Michle. 2010. Protocol for callus and somatic embryogenesis initiation for *Hibiscus sabdariffa L.* (Malvaceae): Influence of explant type, sugar and plant growth regulator. *Australian J. Crop Sci.*, 4(2): 98-106.

Ruan, C.J., X. Zheng, A. Jaime, T.D. Silva and P. Qin. 2009. Callus induction and plant regeneration from embryonic axes of *Kosteleletzkya virginica*. *Scientia Horticulturae*, 120(1): 150-155.

Salikhov, S.A and Z.K.H. Khabibov. 1979. Some phytochemical characteristics of Holly hockc. *Dokl. Akad. NAuk Uzb. Ssr*., 6: 47-48.

Salikhov, S.A. and U.M. Idriskhodzhaev. 1978. (Inst. Bot., Tashkent, USSR). Prospective colouring plant for food industry. Khlebopek, Konditer, Prom-st. (8): 23-4.

Shome, U., S. Mehrotra and H.P. Sharma. 1992. Comparative Pharmacognosy of Two *Althaea* spp. and "Gulkhairo" Samples. *Pharma Biol*., 3(1): 47-55.

Sie, R.S., G. Charles, H.F. Sakhanokho, Y. Toueix, Y. Djei, A. Sangare and M. Branchard. 2010. Protocols for callus and somatic embryo initiation for *Hibiscus sabdariffa* L. (Malvaceae): Influence of explant type, sugar, and plant growth regulators. *AJCSI*, 4(2): 98-106.

Srivastava, S.K. and A. Jain. 1984. Pigment from flower of *A. rosea Indian drugs*, 21(10): 468-9.

Stewart, J.M. and C.L. Hsu. 1977. *In vitro* ovule embryo culture and seedling development of cotton (*Gossypiums hirsutum* L.). *Planta*, 137: 113-117.

Still, M.S. 1994. *Manual of Herbaceous Ornamental Plants*. Champaign, Ill: Stipes Publishing.

Takeda, K., S. Enoki , J.B. Harborne and J. Eagles. 1989. Malonated anthocyanins in Malvaceae: Malonyl Malvin from *Malva sylvestris*. *Phytochem*, 28(2): 499-500.

Tamas, M. and S. Stoleriu. 1976. Chromatographic identification of anthocyanidine and anthocyanosides in some flowers and fruits of indigenous plants. *Biochem*., 19(1): 113-19.

Tomoda, M., K. Shimada and N. Shimizu. 1983. Plant mucilage-A representative mucilage, "Althaea mucilage R" from root of *A. rosea*. *Chem. Pharm. Bull*., 31(8): 2677-84.

Triplett, B.A., T.C.S. Moss, J.M. Bland and M.K. Dowd. 2008. Induction of hairy root cultures from *Gossypium hirsutum* and *Gossypium barbadense* to produce gossypol and related compounds. *In vitro cell. Dev. Biol-Plant.*, 44: 508-517.

Yutako, O.V., H. Mizukami, K. Tomita, N. Hiraoka and K. Fujimoto. 1989. Manufacture of anthocyanine pigments by tissue culture of callus. *Jpn. Kokai Tokyo. Kho. Appl*., 87: 656-660. Anthocyanin production in callus cultures of rosselle *H. sabdariffa Incomplete reference*

Zapata, C., M. Srivatanakul. S.H. Park. B.M. Lee. M.G. Salas and R.H. Smith.1999. Improvements in shoot apex regeneration of two fibre crops: cotton and kenaf *Plant Cell, Tissue and Organ Culture*., 56: 185-191.

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