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Regeneration of Cotton (*Gossypium hirsutum L.*) Through Asexual Methods, A Review

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Abstract: Cotton being a major cash and oil crop has a significant role in the industry and economy of the Pakistan so improved cultivars of the upland cotton is the main focus of the plant breeders. In tissue culture new plants have been developed from different sources like hypocotyls, cotyledon, root, anther of various species but efficient *in vitro* techniques are limited in cotton as compared to other major crops. Factors affecting the tissue culture response in *Gossypium* are genotype, donor plant, growth regulators, type of sugar, culture medium, temperature and subculture timing. The basic pH formulation is suitable to obtain the vigorous callus, it is also observed that the specificity of the culture medium influence the callus initiation and growth. There is a large difference in the ability to form callus between the different genotypes of the cotton, Coker was the 1st genotype that show the callus initiation and regeneration.

Key words: Cotton · Stem Culture · Ovule Culture · Protoplast Culture · Somatic Hybridization

INTRODUCTION

Cotton is a major cash and oil crop of Pakistan. It has given a big boost to the economy and agricultural industry. Up to 70 % domestic edible oil production is also produced by cotton. Cotton is considered recalcitrant to *In vitro* proliferation [1]. Somatic embryogenesis and plant regeneration has been reported from hypocotyls [2-4], but the response is highly genotype dependant [5-7]. Cotton plants are very limited in their regeneration *In vitro* from callus, protoplast or leaf tissues. This widespread problem presently restricts improvement of the few potential commercial genotypes through genetic engineering [8, 9]. Development of tissue culture protocols to induce efficient proliferation in a genotype independent manner is desirable for genetic transformation of cotton. The methods of producing somatic cell hybrids have opened up exciting possibilities for genetic studies concerning the development of improved agricultural varieties [2,10-13].

The first significant work in cotton callogenesis was reported by Beasly [14], who successfully induced callus from ovule. Three years later, cotyledon achieved the induction of callogenesis from cotton [15]. Since then, comprehensive studies are conducted on callogenesis from several cultivars using various explants, growth regulator combinations and carbohydrate source [2,13,16-18].

Regeneration of Cotton: Plant tissue culture is a method of culturing isolated plant organs, tissues, cells and protoplasts in an artificial medium to regenerate into a new plant, which includes somatic cell culture, protoplast culture and somatic hybridization, shoot tip culture and anther or pollen culture [19]. Cotton tissue culture is the basis of cotton genetic engineering and plant regeneration is the first and the most important step in genetic improvement. An efficient regeneration system is a prerequisite for genetic transformation of plants [6]. Cotton tissue and somatic culture has developed rapidly

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since Beasley 1971 who was first one to induce the callus from upland cotton. Several scientists have successfully produced somatic embryoids and multiple shoots using various methods and media from somatic tissues of cotton plants [4,12,20-23]. This made a good foundation for the cotton genetic engineering [24]. *In vitro* cultured cotton cells have been induced to undergo somatic embryogenesis in numerous laboratories using varied strategies [4, 12, 20, 22-26]. Regenerated plants have been obtained from explants such as hypocotyl, cotyledon, root [27], anther [28] and from various cotton species [29, 23]. Somatic embryogenesis and plant regeneration systems have been established from cotton tissue, protoplasts and ovules [30]. Regeneration procedures have been used to obtain genetically modified plants after *Agrobacterium*-mediated transformation of hypocotyls [31, 32] and cotyledons [6] or by transformation of particle bombardment [33, 34]. Efficient *In vitro* techniques for the regeneration of large number of the plantlets for cotton are limited when compared with other major commercial crops. Following are the different sources used in cotton tissue culture as explants.

Cotton Stem-Tip Culture: To date, only few reports of high frequency regeneration in cotton somatic embryos are available due to genotype dependent response [35]. In the same way cotton shoot organogenesis through callus induction has its own difficulties including the excretion of secondary metabolites from the explants into the medium, browning of callus after a short period of culture, a low frequency of organogenic callus formation and very slow response for shoot proliferation from the selected organogenic callus cultures.

Age and size of explant is the most important factor. Shoot tip from less than 5 days old seedlings are difficult to isolate, due to small size and tenderness [36]. Likewise it was also difficult to excise apices of more than 10 days old seedlings due to maturity and hardness of tissues, because if the tissues become more mature, hard or woody, the phenolic compounds will be difficult to control [37]. According to Rashid *et. al.* [36] 6.0 mm size shoot apex from 10 days old seedling showed best response for shoot and root formation on MS basal media and the response of different varieties was 12-100% for plant formation. In other experiments smaller shoot apex size was taken but in those cases seedling age was also less than 10 days [1,8, 37, 38].

Cotton Ovule and Embryo Culture: Ovule culture has received the most attention and has been used for a wider range of objectives than any other aspect of *In vitro* culture of cotton. The first reported attempt to culture ovules was by Joshi [39]. Six DPA (days post anthesis) ovules were excised and cultured on a low salt medium. There are many excellent traits in the wild cotton species and sexual crossing is the common method to transform these traits from wild cotton species to cultivated cotton. However, due to the distance of genetic relationship between the wild cotton species and upland cotton, the inter-specific hybrid seeds are difficult to obtain, mainly because of the abnormal development of the hybrid endosperm. Furthermore, embryo culture *In vitro* is an important experiment method to study the fertilization. In China, Qian and Liu [19] obtained a number of hybrids between cultivated species, *G. hirsutum* and *G. arboreum* and many diploid wild species via culture of interspecific hybrid embryos on the white medium. Up to now, many interspecific hybrid plants including *G. hirsutum* × *G. arboreum, G. hirsutum* ×*G. thurberi, G. hirsutum* × *G.klotzschianum, G. hirsutum* × *G. bicki, G. arboreum* × *G. harknessii, G. herbaceum* × *G.armourianum* and *G. barbadense* × *G. australe* have been obtained and many new germplasm with good abilities of disease resistance, insect resistance, drought and salt tolerance etc. have been derived from these interspecific hybrids and some of them have been widely applied in breeding program [40].

Normally an upland cotton ovule has about up to 32,000 fiber cells out of them 30% dividing within the 1st 72 hour of the culture. Thus time of the transfer of the ovule after post anthesis to culture is most important [41].

Cotton Somatic Culture: Somatic embryogenesis is one of the basic tools widely used in the research of crop biotechnology. It is useful for micro- propagation and for genetic manipulations of plants, which can be used for raising fully transformed plants after mutagenesis or gene transfer. The somatic embryos can be produced with high frequency, but maturation and conversion into plants are still a tedious task, requiring optimization of medium and environmental conditions. However regeneration via somatic embryogenesis in cotton is a challenge due to: 1) genotype dependence, 2) somaclonal variation, 3) lack of knowledge about inheritance and gene action during *In vitro* embryogenesis and 4) specific media requirements

by different genotype [42]. In cotton, somatic embryogenesis has been mostly restricted to *Gossypium hirsutum*, while there are only few reports of somatic embryogenesis mediated regeneration in other cotton species

Protoplast Culture and Somatic Hybridization: Somatic hybridization via protoplast fusion makes it possible to combine the good traits from different species, which is very difficult in sexual hybridization. She *et. al.* [43] was the first one to obtain successfully the regenerated plantlets from protoplast culture of upland cotton. Li *et al.* [31] and Meng *et al.* [44] obtained the embryos from protoplast culture of more than 20 cultivars of *G. barbadenese* like Xinhai 3, Xinhai 6, Xinhai 7, 282, K253, Junhai 1 and Giza 70 etc, through the establishment of embryogenesis somatic suspensions. Wang *et. al.* [45] isolated the protoplast from the embryonic callus of upland cotton, Lumain 6 and the regeneration plants were obtained after several subculture of protoplasm culture. Lv *et. al.* [46] regenerated plants from protoplasts of upland Cotton var. Coker 201. They indicated that IAA and 2, 4-D had a positive effect on callus induction of protoplast culture, 2, 4-D was stronger than IAA, but KT showed a negative effect on protoplast culture. All those plant growth regulators had negative effect on embryogenic callus suspension culture. Due to the success of cotton protoplast culture, somatic hybridization was carried out successfully in China. Sun *et al.* [47] obtained 18 symmetric somatic hybrid plants between *G. hirsutum* var. Coker 201 and *G. klotzschianum* by the method of electrofusion, In the succeeding year, they isolated protoplasts from different explants, which were cultured on liquid KM8P medium. And finally the hybrid plants between upland cotton (Coker 201) and *G. klotzschianum* were regenerated by the protoplast fusions and somatic embryogenesis [28, 48]. Fu *et al.* [49] tried to make the non-symmetric somatic hybridization in cotton protoplast fusion using the technique of inactivation both donor and recipient parent protoplasts and fusion by electricity and the hybrid plants were regenerated from upland cotton (YZ-1) and *G. davidsonii*. It was a novel progress in somatic hybridization following the symmetric fusion using the asymmetric fusion technique based on UV radiation.

Cotton Anther Culture and Haploid Breeding: Anther culture is an important method to obtain haploid plants, which can be used to get stable and genetically homozygous diploid plants through the chromosome doubling. Resultantly, the period of breeding program can be shortened greatly with high efficiency in selection using haploid breeding method. Also, the population with doubled haploid plant lines is a valuable population in theoretical research of plant hereditary and QTL mapping, as well as the studies of differentiation of cotton pollens or microspores and the mechanism for formation of somatic embryo. There are only a few publications on cotton anther culture. Zhang *et. al.* [50] reported the process and morphological characteristics of embryogenesis and organ differentiation in the anther culture of *G. klotzschianum* and the differentiated embryoids, adventitious buds and the haploid plants from the anther calli of *G. klotzschianum* were obtained [30, 25]. However, there are no any reports on the other culture of upland cotton up to now. The key problem of upland cotton anther culture is the division of the pollens or microspores inside the anther. Many factors such as genotypes, developing time of anther, medium and its composition and culture method etc. may affect the anther culture. It was concluded that the technologies of cotton anther culture and inducing of cotton haploid line are valuable in the research of cotton biotechnology, especially for the study of differentiation of cotton pollens or microspores and the mechanism for formation of somatic embryo.

Factors Affecting Cotton Regeneration: The main factors determining the tissue culture response in cotton and other recalcitrant crops include genotype [51], donor plant [52], type of growth regulators [53, 17], sugar type [54] culture medium [55] and culture subculture timing [23]. An in-depth study of such factors would enable the development of genotype-specific culture methods to enhance the tissue culture response of the recalcitrant crops.

Culture Conditions and Temperature: Among the physical factors, Zhang and Wang 1989 found that the suitable temperature for somatic culture was 28-30°C, when the temperature was below 25°C, the callus grew slowly and was difficult to induce somatic embryos. He

further noticed that the temperature above 30°C could easily accelerate aging of callus. Also other factors could play important role in somatic culture, such as the culture condition for germ-free seedlings, explant types and explant age etc.

Hormones: Hormone is an important factor that affects the induction and growth of callus as well as the embryogenesis [56]. For the auxins, 2, 4-D is the most effective in order to pinduce both non-embryogenic and embryogenic callus. However, 2, 4-D may inhibit severely the embryogenic callus differentiating. In a medium with 2, 4-D, it is very difficult to form embryoid from callus, even if in a low concentration. Thus the medium for embryoid growth should be supplied with IAA instead of 2, 4-D [57]. IAA is very efficient in inducing embryoids from embryogenic callus, while NAA is poor in embryoid inducing [58]. BR can extend the explant survival time and it is good in cotton somatic culture when BR combined with other plant growth regulators. For the cytokinin, KT is better than the 6-BA and normally the combination of auxin and cytokinin is suitable for callus induction and embryogenic formation during the cotton somatic culture [56, 23].

A range of hormone regimes were tested by Rashid *et al.* [36] for callus initiation via hypocotyl, cotyledon and root segments. Different based on hormone regimes and nature of the explant was observed. The induction percentage of callus initiation and dry weight of callus formed were increased with 0.1 mg/l 2,4-D + 0.5 mg/l KIN (M3). Although 0.1 mg/l 2,4-D + 0.1 mg/l ZEA (M5) could induce explant to produce callus, the induced callus turned brown. Furthermore, low concentration of 2,4-D and high concentration of KIN stimulated the proliferation of cotton callus, but NAA and ZEA ones were disadvantageous. In previous reports, 2,4-D was an essential hormone for the induction of callogenesis in cotton and other plants [2, 10, 53, 56, 59, 60].

Zouzou *et al.* [13] observed that auxin [4] or cytokinin [18] is necessary to obtain callus. Whether this is true for certain *Gossypium* species, it should be noted that auxin and cytokinin combination is suitable to obtain more vigorous and friable callus.

Culture Subculture Time: Culture time is also very important in the callus induction and somatic embryogenesis. Zhang and Wang [23] reported that the optimal time for first subculture was 40 days. When the explant cultured on the medium for 20 to 30 days, the callus was too small to die. While somatic embryos subculture for a long period on solid medium, the viability may become poor and it was difficult to regenerate anymore. The culture method of "solid-liquid alternative culture" is good one in subculture, which can maintain the callus viability for relative long period [23]. Wang *et. al.* [61] found that embryogenic callus of upland cotton would change from light-green or gray loose particles into yellowish compacted lump form and embryogenesis was increased with the increasing of the culture period, but decreased when the subculture was done after a longer period. After two and half years of culture or subculture, three different calli were isolated. The first callus could produce a large number of embryoids, the second one could produce a small amount of embryoids and the third one lost the ability of embryogenesis. Based on the experiment, they established a high frequency somatic embryogenesis system for several upland cotton cultivars such as Lumain 6 and Lu 1024.

Composition of the Culture Medium: One of the most important factors governing the callogenesis is the composition of the culture medium. Several media formulations are commonly used for the majority of all cell and tissue culture work. These media formulations were described by Murashige and Skoog [62], Gamborg *et al.* [63], McCown [9]. Murashige and Skoog's medium (MS) and Gamborg's medium (B5) are all highly concentrated in macronutrients, while McCown's medium (MC) formulation contains less of macronutrients. There was significant difference between calli formed among these media. MS medium shows the highest percentage and dry weight callus followed by B5 medium and MC medium which had the lowest. This basic formulation is suitable to obtain vigorous callus [64]. This reactivity difference of MS medium seems to be in relation with the calcium and nitrogen concentrations. In effect, calcium and nitrogen level in MS medium was respectively eight times and four times more important than B5 medium [65]. It was reported that this specificity of each medium can influence callus initiation and growth [16, 13]. Inorganic nitrogen has a determining action on callogenesis [66, 17].

Genotype: Genotype is another major factor that affects callus induction and embryogenesis. Zhang *et. al.* [24] indicated that there was a large difference in the ability of

embryogenesis in 50 upland cotton cultivars. Among the introduced foreign cultivars or germplasms, Coker pedigree germplasms can easily regenerate and that of Acala and Stoneville cotton are more difficult ones in plant regeneration. The cultivars from Deltapine Land Co. are hard to regenerate.

Response in tissue culture is highly genotype dependent. Significant genotypic differences in callus initiation response were observed by Zouzou *et. al.* [65] among the ten cotton genotypes. The same positive response of callogenesis was already reported in other genotypes viz. Coker [16, 7] and Simian [18]. Dry weight which represents the mass of cells produced could be considered as good index of cotton callogenesis [5, 67, 13]. Callogenesis positive response of R405-2000 compared to the others cotton genotypes seem to be related to the differential sensitivity of tissue to callogenesis medium. Similarly, several authors mentioned the influence of the genotype in cotton callus initiation [11, 13, 16, 53, 68, 69]. These genotypic differences with respect to callus initiation were also observed in many other plants [10, 59, 60].

Sugar Type: Sugar influence cells proliferation and differentiation according to Swankar *et. al.* [70] however, sugar doesn't have the same effects on callogenesis. Studies showed that the percentage of induction and dry weigh of callus are more significant with glucose followed by fructose and sucrose. Glucose and maltose showed lowest results [64]. These results were comparable with those showing that glucose product friable and voluminous callus compared to other sugars [65]. The beneficial effect of glucose on callogenesis has also been mentioned in many plants [10, 53, 67, 71, 68]. Indeed, glucose is the assimilated form of sugars by plant cells and the most important source of energy production [72]. Fructose is also an assimilated sugar by plant cells but its reactivity seems to be less compared to glucose. Sucrose is an important biological reservoir for the two previous cited sugars. Sucrose is an analogous of glucose as regards to the physico-chemical properties but, with a low reactivity. An acid medium (pH 5.8), hydrolyses this sugar and breaks it into glucose and fructose which are assimilated by plant cells. They were probably a competition between these two sugars for their assimilation by the cells that makes sucrose less active and consequently less auspicious to cotton callogenesis. Our results showed that maltose and galactose have no beneficial effect on callogenesis, certainly because these two sugars are non assimiliable forms by plant cells. We found that glucose medium inhibited browning in agreement with works of some researchers [73]. With glucose less browning and better callus proliferation occurred. Consequently, glucose is sugar which induced the better response to cotton callogenesis.

However, callus dry weight analysis revealed that 4% of glucose produced highest callus dry weight followed by 3% of glucose and 5% of glucose. This glucose level seems to provide an adequate osmotic pressure that would permit absorption of mineral nutrients presents in medium which according to several authors are essential to cells growth [74, 19]. Several types of callus were distinguishable based on the physical appearance under different levels of glucose. Calli on medium containing 1 and 2% of glucose were green and compact, whereas those coming from 3 and 6% of glucose were green yellow, friable and browned. In contrast, calli from medium supplemented with 4 or 5% of glucose were green grayish, friable and no necrosis. These morphologic observations are characteristic of embryogenic structure induction according several authors [16, 75, 76]

Donor Plant/ Explant: Explant type seems to play a significant role in cotton callus initiation. Variation in callus forming ability of different explant types has been reported in many others plants [71, 77]. Callogenesis specificity of explant type would be explained by their differential reactivity to media components [78, 16, 65]. Sugar influence cells proliferation and differentiation according to Swankar *et. al.* [70].

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