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Micropropagation of *Lilium Asiatic* in an Efficient Low Cost Novel Medium "KFA and KFA plus"

Kakoli Biswas[1*] and Rajesh Biswas[2*]

[1] Department of Biotechnology, DAV College, Sector-10, Chandigarh, India.
[2] Department of Zoology, Government Home Science College, Sector-10, Chandigarh, India.
** Corresponding authors email ID : rajeshbiswas63@yahoo.co.in, kakolibiswas14@gmail.com*

Abstract

Low cost technology for Plant Tissue Culture is promoted worldwide especially for the production of flowering or ornamentals crops like *Lillium asiatic*. As tissue culture media plays an important factor in deciding the cost of *in vitro* plants a continuous quest to find the substitutes of media components is in process. In an attempt to lower the cost of the in vitro micropropagated plants of different varieties of exotic *Lillium asiatic,* we developed an efficient low cost medium "KFA and KFA plus" (Flyash" as the main source of inorganic constituent; Patented), which could replace the widely used expensive Murashige and Skoog's medium. The comparison was done on four major criteria: % bud break, % shooting, % rooting and cost. When cultured on KFA and KFA plus, 70% bulblet formation was observed in KFA and 86.6% bulblet formation in KFA plus as compared to 83.3% in MS medium supplemented with IAA (0.8mg/l) + BAP (1.5mg/l). Healthy response and an average of 1.6 bulblets/explant were obtained. Healthy rooting in 70% bulblets cultured on KFA plus medium supplemented with same concentration of PGR was observed as compared to 50% rooting in MS medium. Cost of media was reduced 10 times by using KFA plus as culture media as compared to MS ready media (Hi media, India) and very encouraging results in relation to growth and multiplication was obtained. Therefore, use of flyash media resolves our main aim to produce low cost plants as well as the reduction of disposal problem of thermal power plant waste, leading to phytoremediation.

Keywords: Novel medium, flyash, Plant tissue culture, *Lillium asiatic*, KFA and KFA plus, micropropagation, phytoremediation.

1. INTRODUCTION

Ornamental plants have a huge market worldwide and hence require continuous production to fulfill the demand and supply ratio. Micropropagation through tissue culture is one of the best options for large scale production from single individual tiny stem cuttings, axillary buds, bulbs etc. to achieve rapid proliferation in short time and limited space thus maintaining a continuous supply. However at large scale, plant tissue culture becomes expensive and therefore there arises a need to reduce the cost of the regenerated plants. Low cost technology for Plant Tissue Culture is promoted worldwide especially for the production of flowering or ornamentals crops like *Lillium asiatic*. As tissue culture medium plays an important factor in deciding the cost of *in vitro* plants a continuous quest to find the substitutes of media components is in process.

Mineral salts and sugar as carbon source and water are the main components of a culture medium. Other components include organic supplements, growth regulators, a gelling agent[1,2]. Various types of starches and plant gums as cheaper alternatives to agar were used[3,4]. Tyagi *et al* [5] replaced laboratory grade sucrose by locally available commercial sugar as carbon source and bacteriological grade agar by isabgol as gelling agent. Table sugar as an alternative low cost medium component for *in vitro* micro propagation of potato (*Solanum tuberosum* L.) was investigated [6] by Demo, *et al.*

In an attempt to reduce the cost of the in vitro micropropagated plants, we developed an efficient low cost medium "Kakoli Fly Ash" (KFA and KFA plus)[7] based on Fly Ash (FA) which have potential to replace the widely used expensive Murashige and Skoog's (MS) medium for large scale production. KFA and KFA plus was used for micropropagation of Mentha sps.[8]. In general, 95-98% of FA consists oxides of Si, Al, Ca and about 0.5-3.5% of Na, P, and S [9] revealed useful ameliorant nature of FA, improves properties of problem soils [10]. It is a source of readily available plant macronutrients like K, P, Ca, Mg, S and micronutrients like Fe, Zn, Cu, Mo, B, Mn. In the present study it was used in various combinations with and without nitrogen source, and plant growth regulators.

2. MATERIALS AND METHODS

2.1 Source of Explant material:

Bulbs of different varieties of *Lillium asiatic* were procured from Dr. Y. S. Parmar Horticulture University, Nauni, Solan, Himachal Pradesh.

2.2 Subject studied:

Fly ash used in study was collected from the dumps of thermal power plant, grey in colour and having pH-8.0

2.3 Establishment of culture:

Aseptic cultures were established from the scales of bulbs containing meristematic tissue. The explants were washed thoroughly with water to remove the dirt and surface sterilized with 70% ethanol for 30 seconds followed by 0.1% (w/v) mercuric chloride for 7 minutes followed by rinsing with sterile distilled water thrice and inoculated in KFA, KFA plus which had 10% Flyash (FA) as the main source of inorganic constituent and MS [11] medium which was used as control. KFA plus was supplemented with nitrogen source comprising of Glycine 2mg/l, Nicotinic acid 0.5 mg/l, Pyridoxine-HCl 0.5mg/l, Thyamine-HCl 0.1mg/l while KFA was devoid of nitrogen source. All the three media were supplemented with 3% sucrose (w/v) as carbon source, 0.8% agar (w/v) and plant growth regulators (PGRs) in the combinations/concentrations of NAA-0.5mg/l and FAP-1.0mg/l, IAA-0.8mg/l and BAP-1.5mg/l, IAA-0.25mg/l and FAP-0.5mg/l. The pH of all media was set to 5.7. All cultures were set in ten replicates for each combination and the experiments were repeated thrice. The cultures were maintained at 25±2^{o}C, 1500 lux intensity, photoperiod of 16hrs light/8hrs dark regime and 70% humidity. The cultures were routinely observed for any contamination. Different growth parameters like bud break with respect to number of days after inoculation, % shoot growth, average bulblets/explants, % rooting and morphological characters of regenerated plantlets were recorded routinely. Statistical analysis was carried out by calculating Mean ($\overline{X}$), Standard Deviation (σ) and Standard Error for induction frequencies of the explants and % shooting. Analysis of Variance (ANOVA) was performed for the media type and their response to culture of plants. All statistical analysis was performed by using SPSS software program.

3. RESULTS AND DISCUSSION

3.1 Bulblet and Shoot Formation: Initial cultures of *Lilium asiatic*, were established on MS medium, low cost medium KFA and KFA plus supplemented with all the three combinations of PGRs. When explants were cultured on flyash containing medium, it showed 70% bulblet formation in KFA and 86.6% bulblet formation in KFA plus as compared to 83.3% in MS medium supplemented with IAA (0.8mg/l) + BAP (1.5mg/l) after 8 days of initial cultures (Fig. 1). In media supplemented with NAA (0.5mg/l) + FAP (1.0mg/l), 83.3% bulblets were obtained in KFA plus, 66.6% bulblets in KFA as compared to 73.3% bulblets in MS medium after 12 days of initial cultures. Plants showed healthy response and multiple bulblets formed when further subcultured in the same media with the two best PGR combinations (Plates 1, 2A & 2B). Around 500 bulblets were obtained after 3 subcultures. Only 25% response was observed in IAA-0.25mg/l and FAP-0.5mg/l. Mean bulblet/explant of 2.4 in KFA plus and 1.5 in KFA as compared to 2.2 in MS media with IAA: BAP combination was obtained after 13 days while when supplemented with NAA: FAP combination mean bulblet/explant of 1.6 in KFA plus, 0.9 in KFA as compared to 1.5 in MS medium after 16 days of initial culture was observed (Fig. 2). The bulblets developed in to

shoots after ten days of subculturing in the same medium (Plate.2). Green and healthy shoots developed in all the media supplemented with PGR combination of IAA: BAP.

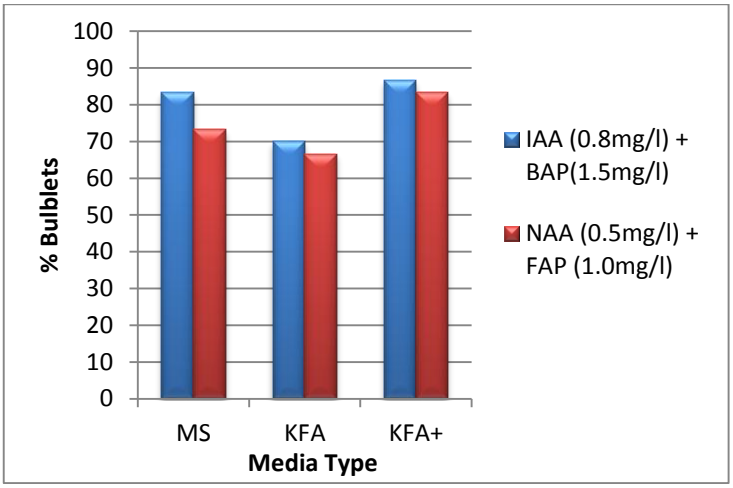

Figure 1: Percentage bulblet formation in *Lilium asiatic* on different media type.

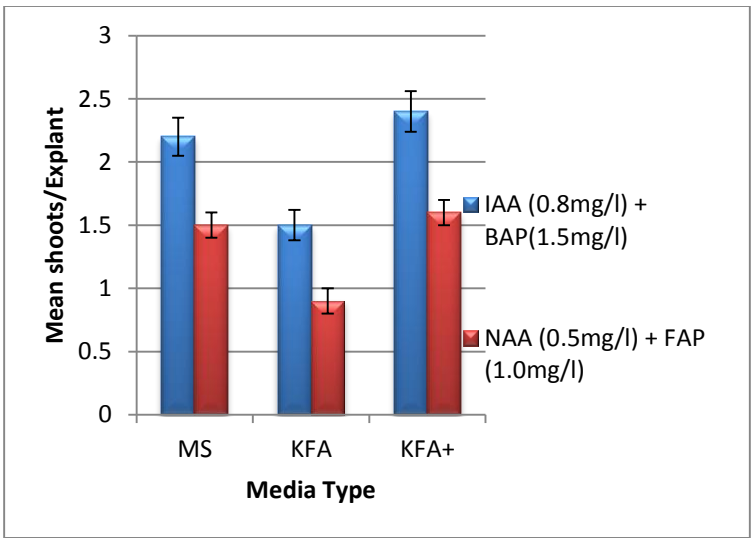

Figure 2. Graph showing Mean bulblet/explant in *Lilium asiatic* on different media type with two different PGR combination.

3.2 Analysis of variance:

Data analysis was performed by using ANOVA test.

3.3 Rooting:

Rooting was observed in all the three media composition supplemented with IAA (1.0mg/l+ BAP (0.8mg/l) hormone combination. In KFA plus, 70% rooting was

observed after 15 days as compared to 50% in MS medium after 17 days, however it took about twenty days to obtain rooting in KFA with a rooting of 30% (Fig.3, Plate.3A, B). Maximum rooting was seen in KFA plus medium. Roots were healthy and creamish-white in color.

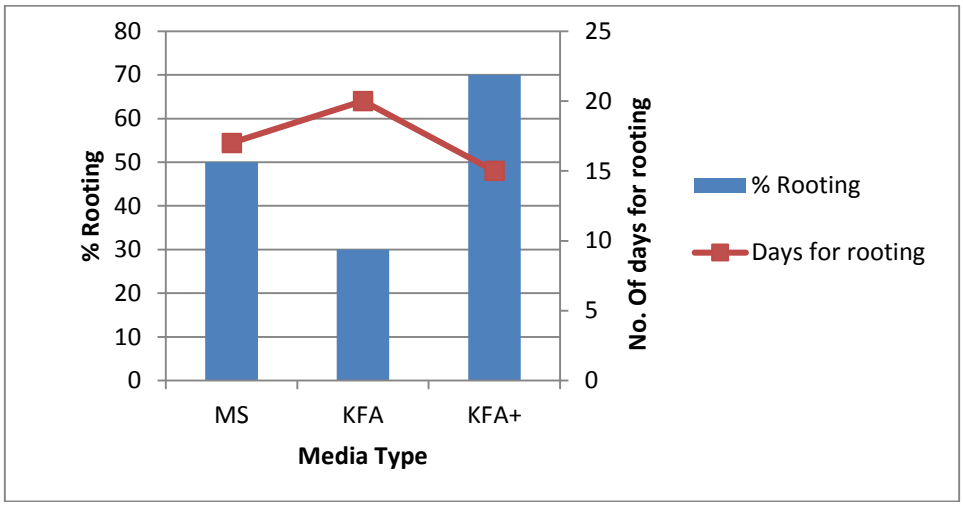

Figure.3: Graph showing percentage rooting in *Lilium asiatic* with time interval (days) on different media types supplemented with IAA (1.0mg/l) + BAP (0.8mg/l).

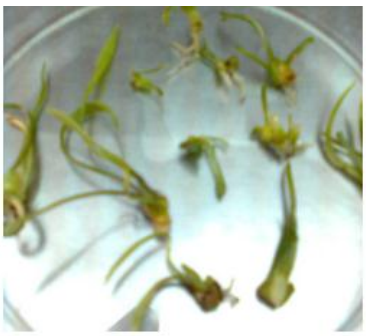

Plate.1

Plate.2A Plate.2B

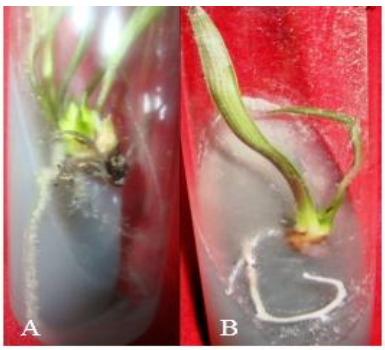

Plate 3

Plate- 1: In vitro derived bulblets of *L.asiatic* on MS and KFA plus media containing IAA (0.8mg/l) + BAP (1.0mg/l).

Plate- 2: Shoots under in vitro conditions on MS (A) and KFA plus (B) media respectively containing IAA (0.8mg/l) + BAP (1.0mg/l).

Plate-3: A & B: Rooting in bulblets of *Lilium asiatic* on KFA plus medium containing IAA(1.0mg/l) + BAP (0.8mg/l).

3.4 Hardening:

Well rooted plants after two weeks of culturing were hardened in special mixture of soil (vermiculite:soil : 1:1). Plants were shifted from high to low humidity and low to high light intensity under sunlight. High survivability rates were observed during hardening (Plate 4A,B).

Number of leaves and length of the plants increases in the winter season and less growth was observed in the summer season

Plate-4A: Healthy buds developed after hardening and **4B:** Flowers blooming in the same plant

3.5 Morphological studies:

No morphological differences were observed in *in vitro* cultured plants. Plants obtained from all different types of media were found similar to those in natural habitat. Leaves obtained were green in color and were healthy. Shape, size, texture and other morphological aspects of explants obtained were exactly similar to natural occurring plants. Ornamental plants have tremendous commercial value. As the main objective of the present study is low cost plant production in tissue culture of an exotic ornamental plant *Lilium asiatic,* the major considerations were to maintain the true to type quality and properties of the plants. With this objective a low cost medium was developed for *in vitro* micropropagation by substituting the inorganic nutrients in the medium with FA to assess its efficacy for large scale plant production.

In a conventional medium, if agar is used as a gelling agent, it can represent up to 70% of the total cost, followed by the minerals, water, sucrose and other minor media components[12]. Earlier several works have been reported related to the reduction in cost of plant tissue culture medium by finding cheaper alternatives for various components of culture medium. Cheaper alternatives to agar include various types of starches and plant gums were used, but no satisfactory results were found[3,4]. Plant did not respond well due to brittleness of the media. Naik and Sarkar [13] **used sago as cheaper gelling agent for potato regeneration.** Alternative for artificial light[14, 15],

tubular skylight[16], the natural-light of a nethouse[17] were used. The use of common sugars reduced the cost of micropropagation medium by 78 to 87% [12]. We have reported the effectiveness and enhanced production of *Lillium* using two alternative sugar sources: Candy sugar (misri) and Sugar cubes[18] .

This was the first time when FA has been used in tissue culture for Lilium. For this novel media the patent has been filed[7]. Two plants were selected based on their ornamental values and the effect of FA was seen and compared with the control MS medium. Cost of the media was reduced 10 times by using FA medium as compared to MS medium.

In *Lilium asiatic*, best response was observed when explants cultured on KFA and KFA plus medium showed 70% and 86.6% bulblet formation respectively, as compared to 83.3% in MS medium when all of them were supplemented with IAA (0.8mg/l) + BAP (1.5mg/l). Previous studies reported highest bulblet formation (78.64%) with different levels of NAA and BAP[19] which was less than that obtained by us in KFA plus media (86.3%) supplemented with IAA: BAP. Similarly, 70% rooting was observed in bulblets cultured on KFA plus medium supplemented with same concentration of PGR [IAA (1.0mg/l+ BAP (0.8mg/l)] as compared to 50% rooting in MS medium. Comparison done by using ANOVA showed significant value (0.250) for *Lilium asiatic* indicating that there is a significant difference between the mean of three variables (MS, Flyash and various hormone combinations) used (Table.1).

Table 1: Showing main ANOVA summary output.

Variables	Sum of Squares	df	Mean Square	F	Sig
Between Groups	3.200	1	3.200	1.412	.250
Within Groups	40.800	18	2.267		
Total	44.000	19			

In our earlier study on micropropagation of *Mentha sps*., using fly ash based KFA and KFA plus culture media, 83.3% shooting and an average of 1.27 shoots/explants was reported [8]. The study also reported the advantage of KFA and KFA plus media over MS media which is in agreement with our present finding.

Based on the obtained results this can be inferred that KFA plus medium was significantly better in comparison to MS medium for production of in vitro plants of *Lilium asiatic*. "KFA plus" medium is also cheaper than MS medium thus providing an advantage over MS medium. The reduced response in KFA may be attributed to the absence of nitrogen source and is very important for *in vitro* propagation as N_2 is required for synthesis of amino acids and nitrogenous bases which are the building blocks for proteins and nucleic acids respectively. However since this medium too showed growth can be used as medium.

Another important concern about Fly ash, a waste product of the thermal power plant, is its disposal, thereby posing threat to the environment due to its easy dispersal and remaining as suspended particles in the air causing respiratory problems. Therefore, use of flyash based media can solve dual problem by fulfilling the aim to produce low cost plant production as well as the disposal problem of thermal power plant, leading to phytoremediation. Plant tissue culture on large scale is very expensive; therefore it is the need of the day that some alternative has to be found out. In the present study FA was used as alternative medium for Lillium. Cost of the FA based medium was less; almost negligible as compared to MS medium. Growth and multiplication was also superior in FA based medium as compared to MS medium. Therefore FA based medium providing an advantage over MS medium to be substituted as culture media in order to make it cost effective for Lillium and probable candidate for substitution for many more plants.

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