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Optimization of conditions for callus induction and indirect organogenesis of *Cucumis anguria* L.

[1]J. Jerome Jeyakumar[*] and [2]L. Vivekanandan

[1]Department of Biotechnology, Pavendar Bharathidasan College of Arts & Science, Mathur, Pudukottai Road, Tiruchchirappalli, Tamil Nadu, India
[2]Plant Physiology and Biotechnology Division, UPASI TRF, Tea Research Institute, Nirar Dam P.O., Valparai, Tamil Nadu, India

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ABSTRACT

An efficient procedure has been developed for callus induction and indirect organogenesis of Cucumis anguria L. Seedlings were grown under in vitro conditions and selection was carried out between the leaf and nodal segments for the explants in the seedlings. Different parameters like the explant source, hormones and sugars were checked for the optimization of cultures. Leaf explants which showed the maximum response to callus induction (42.0%) was thus, selected for the study. MS medium supplemented with 4.0 mg L^{-1} 2,4-D and 4.0 mg L^{-1} NAA proved to be the most efficient hormone in promoting callus development from the leaf explants with 42.0% response and was the most favorable medium. Apart from the type of explant and growth regulators, different sugars and their concentrations were also checked for the induction of callus. 3% glucose produced highest rate of callus in the culture. In response to MS medium with 3.0 mg L^{-1} BAP and 0.5 mg L^{-1} Kn, best response of shoot induction was evoked with an average number of 5.02±0.76 shoots per callus. In vitro regenerated shoots (3.0 cm long) were excised aseptically and implanted on MS half strength medium fortified with 3.0 mg L^{-1} NAA and 2.5 mg L^{-1} IBA. Following initial hardening for four weeks, the rooted plantlets were successfully acclimatized in pots containing sterilized soil and sand mixture (1:1) with 75% survival rate in the field conditions. The protocol provides a basis for germplasm conservation and for further investigation of bioactive constituents of this medicinal plant.

Keywords: *Cucumis anguria*, Medicinal plant, Callus induction, Shoot proliferation, Indirect organogenesis.

INTRODUCTION

Cucumis anguria L., also known as West Indian gherkhin and belonging to the family cucurbitaceae, is a highly nutrient vegetable with traditionally medicinal plant, mainly cultivated and consumed in Africa, Brazil, Cuba, India, United States and Zimbabwe [15]. This species is receiving increasing attention as it is commonly used in indigenous and traditional folk medicine system especially in Asia and Africa. The fruit of the vegetable contains high amounts of protein, calcium, phosphorous, iron and vitamin C [29]. The plant is also known for its usage in traditional system of medicine to treat stomach ache, jaundice, hemorrhoids and preventing stone formation in kidney [2] [22]. *C. anguria* consist of many useful compounds such as flavonoids, tannins, alkaloids, saponins and steroids which contains high levels of antioxidant activity [8]. Phytochemists have isolated a number of potential medical components from *C. anguria*, such as cucurbitacin B, cucurbitacin D and cucurbitacins G [24]. Cucurbitacins B have the potential to be used as a favorable phytochemical for cancer prevention. Anthraquinones and saponins which are present in *C. anguria* are used for antibacterial and antifungal activity against clinical pathogens [23]. The roots of this plant are a source of pharmaceuticals, agrochemicals, flavors, dyes, fragrances and many other special chemicals.

Due to continuous overexploitation, this plant has become extinct and there is a danger of losing germplasm of these important bio-resources. There is also an urgent need for the conservation and rapid propagation of this plant.

Medicinal plant cultivations are being considered as an alternative to agricultural processes for producing valuable phytochemicals. Since many of these products are obtained by direct extraction from plants grown in natural habitat, several factors can alter their yield. *In vitro* culture has become an alternative for the production of secondary metabolites. The development of an *in vitro* culture system would offer unique opportunities for producing drugs in the laboratory without having to depend on field cultivation (Sudha and Seeni, 2001) as the system uses explants from mature field grown plants. Development of *in vitro* procedures helps in establishing tissues for germ plasm conservation, rapid propagation and for secondary metabolite production to meet the vastly increasing demand of therapeutic and other industries.

This experiment was carried out to study the growth response of leaf and nodal explants of *C. anguria* under different combinations of plant growth regulators under *in vitro* conditions and for the development of a protocol for production of plants with the objective of facilitating healthy seedlings to gherkin growers and to benefit conservation and regeneration of this important taxon. The present study reports a very simple and reliable protocol for initiation, multiplication and elongation of shoot and rooting of gherkhin via indirect organogenesis from healthy leaf explants to meet these objectives.

MATERIALS AND METHODS

Plant Material

The present work was carried out at Jamal Mohamed College, Tiruchirappalli, India. Seeds of *C. anguria* were purchased from Nunhems Seeds Pvt., Ltd., Bangalore, India. Different concentrations of sugars and hormones were assessed in separate experiments using factorial design plot with 20 replications to decrease the error and enhance accuracy.

Surface sterilization

The seeds were soaked in tap water for 1 h and washed with 10% (v/v) commercial detergent Tween 20 for 10 min followed by 3 rinses with distilled water. Seeds were further disinfected with 0.1 % (w/v) mercuric chloride solution for 5 min and rinsed 5 times with sterile distilled water to remove traces of mercuric chloride. Finally, the seeds were blotted dry on sterile Whatmann No.1 filter paper. Disinfected seeds were placed in test tubes (25 × 150 mm) containing sterile moist cotton and plugged tightly with non-absorbent cotton and placed in dark for germination.

Culture conditions

Stock solutions of MS media used in this study were prepared by dissolving the constituents of salt, iron and vitamin solution in 1L of distilled water and kept in dark-colored bottles in a refrigerator [17]. The media were further augmented with various concentrations and combinations of growth regulators namely BAP, and IBA. The medium pH was adjusted to 5.7 before solidifying with 0.7 % (w/v) agar. The melted medium was dispersed into 50 mL test tubes at 10 mL per tube and plugged with non-absorbent cotton. Culture tubes containing the media were autoclaved at 121°C for 20 min with 15 lbs pressure. Explants were inoculated on the culture medium containing various growth hormonal combinations and transferred to a growth room for maintenance at 20 ± 2 °C and 16 h photoperiod and 8 h dark period. Light was supplemented using white fluorescent tube at a photosynthetic photon flux density of 40-45 µmol m^{-2} sec^{-1}.

Callus induction

Effect of growth regulators and explant type

Various concentrations of the hormone solutions were prepared and added to the culture medium. Twelve combinations of growth regulators which included 2,4-D and NAA separately and combined with each other were tested. Leaf pieces (16~25 mm^2 surface area) and nodal segments (6 mm length) of 7- day-old sterile seedlings were used as explant type. Calli were harvested and weighed for fresh weight determination. All the explants were maintained for 3 weeks in a growth room at 22 °C, 70% humidity and 16 h light photoperiod provided by cold fluorescent lamps.

Effect of sugar types and concentration

The hormone regime which showed the best callogenesis potential expression was retained to study the effect of sugar type on callogenesis. Six sugars (glucose, fructose, galactose, maltose, sucrose and sorbitol) were tested in the study. Six different concentrations of the sugar which could promote callus production (1%, 2%, 3%, 4%, 5% and 6%) were also tested to identify the level of sugar. All the explants were incubated in Erlenmeyer flasks, under the same culture conditions. Over 4 week's period, the ability of the seedlings to develop callus on the above media was observed.

Plantlet establishment

Small bits of calli were excised from 20-days old callus cultures and cultured on MS medium supplemented with BAP (1.0-5.0 mg L^{-1}) in combination with Kn (0.5 mg L^{-1}) for multiple shoot induction. The shoots obtained from the callus were transferred on to fresh medium. Subsequently, shoots elongated were repeatedly sub-cultured at three week intervals on the same media for further shoot elongation and proliferation.

Elongated shoots (>2cm) that possessed approximately 3 compound leaves were cultured on half-strength MS medium with supplemented with NAA (1.0-5.0 mg L^{-1}) and IBA (0.5–2.5 mg L^{-1}). Well developed *in vitro* rooted plantlets were removed from the culture medium and the residual agar were removed by washing the roots under running tap water followed by transferring them to plastic cups containing garden soil and sterile sand in the ratio 1:1. The cups were covered with polyethylene bags and kept inside the tissue culture room to maintain humidity condition. The plantlets were maintained under high humidity for 4 weeks by spraying liquid half strength MS medium every alternate day. They were slowly weaned to lower humidity by making small holes around the polyethylene bags of potted plantlets for a period of further 4 weeks. The plantlets were transferred to pots containing garden soil with organic manure and kept in the green house condition for another 4 weeks. All the surviving plants were transferred to green house by removing the polyethylene bags before exposing them to the experimental field under natural conditions.

Statistical Analysis

All the experiments described above were conducted in a completely randomized design and repeated twice. Means and standard error of means for all dependent variables such as callus induction, shoot regeneration, shoot number and shoot length under different plant growth regulator concentrations were computed and determined the significant differences between means using DMRT.

RESULTS AND DISCUSSION

Initiation and Establishment of cultures

Mature seeds of *C. anguria* were germinated under *in vitro* conditions. From the 7-days old *in vitro* seedlings, leaf pieces and nodal segments were excised and used as explant source.

Effects of exogenous plant growth regulators on callus formation

Green compact were induced when low concentrations of 1.0 and 2.0 mg L^{-1} of 2,4-D was used. With more than 2.0 mg L^{-1} 2,4-D, only yellow friable callus was formed. Only the green compact callus was able to regenerate adventitious shoots after being transferred to differentiation medium (Table 1). Calli were successfully generated in all the media containing 2,4-D and NAA alone and in all combinations of two months after inoculation. This indicated that exogenous hormone was essential to callus formation of *C. anguria*. The exclusive presence of NAA in the medium, regardless of its concentration, was less satisfactory for callus initiation in comparison with 2,4-D. However, in the presence of 2,4-D, the cultures containing corresponding concentrations of NAA showed higher callus weight in a range from 0.51 g to 0.83 g FW per explant and higher response from 29.0% to 42.0% (Table 1). This phenomenon suggests that 2,4-D played a more important role in callus formation from leaf explants compared to NAA.

The combinations of auxin and cytokinin were found to produce more callus than auxin or cytokinin alone. Our results show that 2,4-D and NAA was most effective on callus induction. The combination of 2,4-D and NAA induced high callusing frequency at all concentrations studied. A combination containing 2.0 mg L^{-1} 2,4-D and 2.0 mg L^{-1} NAA proved to be most efficient in promoting callus development from leaf explants with 42.0% response, followed by 1.0 mg L^{-1} 2,4-D and 2.0 mg L^{-1} NAA and then 3.0 mg L^{-1} 2,4-D and 2.0 mg L^{-1} NAA. In addition, most of the cultures containing 2.0 mg L^{-1} 2,4-D and 2.0 mg L^{-1} NAA produced green compact calli, which initiated at cut surfaces of the explants after 54 and 58 days of inoculation respectively and proliferated quickly. However, cultures with NAA only resulted in more friable calli and white roots germinated from those compact calli after 75 of cultivation. This observation was similar to callus development of *Holostemma adakodien* [16]. These rooty calli did not regenerate shoots on MS medium containing BA. This is in agreement with an earlier report on switchgrasss that highly rooty calli cannot regenerate shoots [7]. At low concentration (1.0 mg L^{-1}), NAA only resulted in root formation from the midrib. With increased NAA concentration, roots were also induced from cut edges of leaf explants and the rooting rate as well as root number per explant increased. The roots, however, grew slowly at increased NAA concentration. The effect of NAA in combination with 2,4-D on callus induction was studied by increasing 2,4-D concentration while NAA was kept constant, with the result that the rooting rate of leaf explants decreased. It is obvious that 2,4-D inhibited root development induced by NAA and induced callus. This is in contrast with the study in 'Knnow' mandarin and *C. reticulata* where NAA was better in inducing embryogenic callus, while 2,4-D only induces non-embryogenic and friable callus that cannot regenerate shoots [9][10]. Callus

induced from the stems of *C. grandis* could regenerate shoots on differentiation medium, while the morphogenetic pattern of callus tissue shifted from shoot bud differentiation to embryogenesis during prolonged culture on medium containing 2,4-D and NAA. It was suggested that 2,4-D and NAA have different roles on callus induction for various species as well as at different culture stages [6]. In the present study, we have shown that 2,4-D is better than NAA in inducing regenerative callus.

Table 1: Callus induction in *C. anguria* in MS medium supplemented with 2,4-D and NAA

Concentration (in mg L^{-1})	Explants	No. of days for callus induction	Percentage of callogenesis	Texture	Colour
M1: 1.0 mg L^{-1} 2,4-D	Leaf	60	32.6	CP	GR
	Node	65	28.3	CP	GR
M2: 2.0 mg L^{-1} 2,4-D	Leaf	66	35.6	CP	GR
	Node	73	30.5	CP	GR
M3: 3.0 mg L^{-1} 2,4-D	Leaf	64	30.2	FL	YL
	Node	68	26.9	FL	YL
M4: 4.0 mg L^{-1} 2,4-D	Leaf	69	29.3	FL	YL
	Node	74	25.4	FL	YL
M5: 1.0 mg L^{-1} NAA	Leaf	75	24.0	FL	YL
	Node	79	21.9	FL	YL
M6: 2.0 mg L^{-1} NAA	Leaf	72	28.6	FL	YL
	Node	76	24.4	FL	YL
M7: 3.0 mg L^{-1} NAA	Leaf	70	23.0	FL	YL
	Node	74	21.0	FL	YL
M8: 4.0 mg L^{-1} NAA	Leaf	67	21.8	FL	YL
	Node	71	20.5	FL	YL
M9: 1.0 mg L^{-1} 2,4-D + 2.0 mg L^{-1} NAA	Leaf	61	38.5	CP	GR
	Node	65	34.2	CP	GR
M10: 2.0 mg L^{-1} 2,4-D + 2.0 mg L^{-1} NAA	Leaf	54	42.0	CP	GR
	Node	58	39.8	CP	GR
M11: 3.0 mg L^{-1} 2,4-D + 2.0 mg L^{-1} NAA	Leaf	59	34.0	CP	GR
	Node	63	31.0	FL	YL
M12: 4.0 mg L^{-1} 2,4-D + 2.0 mg L^{-1} NAA	Leaf	61	29.0	FL	YL
	Node	63	25.0	FL	YL

CP: Compact; FL: Friable; GR: Green; YL: Yellow;

Table 2: Effect of sugars on *C. anguria* callus culture

Sugar type	Percentage of response	Texture	Colour
Glucose	90	CP	GR
Fructose	85	CP	GR
Sucrose	80	CP	YG
Galactose	40	CP	YG
Maltose	40	CP	BR
Sorbitol	30	CP	BR

CP: Compact; GR: Green; YG: Yellowish green; BR: Brown

Table 3: Effect of glucose on *C. anguria* callus culture

Glucose level (%)	Percentage of response	Texture	Colour
1	10	CP	GR
2	15	CP	GR
3	30	CP	GR
4	40	CP	GR
5	25	CP	GR
6	15	CP	GR

CP: Compact; GR: Green

Effects of different types of explants on callus induction

Explant type and probably its anatomical structure seems to play a significant role in callus initiation in *C.anguria*. The two explants were placed on MS medium with the hormones for 3 weeks of culture. The callusing was different

among the two explants, with leaf callus giving the highest percentage (42%) and the nodal explants giving 39.8% (Table 1). Variation in callus forming ability of different explant types has been reported in many others plants [11], [30]. The results obtained indicated that leaf explants were more suitable for callus induction of *C. anguria* than the nodal explants. Owing to the capacity of higher callus formation, hence, leaf calli were used for further experiments.

Table 4. Multiple shoot induction in *C. anguria*

BAP	KIN	Mean no. of shoots	Shoot length
1.0	0.5	5.60^{d}	3.85±1.1^{d}
2.0	0.5	7.00^{b}	6.32±1.2^{a}
3.0	0.5	8.70^{a}	5.27±1.3^{b}
4.0	0.5	7.20^{b}	4.58±1.4^{c}
5.0	0.5	6.10^{c}	3.89±1.5^{d}

Table 5. Root induction in *C. anguria*

NAA	IBA	Mean no. of roots	Root length
1.0	0.5	5.70^{d}	3.23±0.25^{c}
2.0	1.0	8.70^{b}	3.97±0.80^{b}
3.0	1.5	10.15^{a}	4.29±0.25^{a}
4.0	2.0	8.20^{b}	3.75±0.25^{b}
5.0	2.5	6.45^{c}	3.31±0.25^{c}

Effect of sugars on in vitro callus induction

Sugars influence cells proliferation and differentiation [27]. However, sugars do not have the same effects on callogenesis. The present study shows that the percentage of callus induction is more significant with glucose followed by fructose. The callus initiated on margins from leaves and obtained yellowish green in color and embryogenic which later turned green. After removal of the mother tissue on the first subculture, the callus could be maintained without any morphological changes on many subcultures each spanning 3 weeks. Sucrose in the medium supported the growth of yellowish green callus. Galactose in the medium also gave rise to yellowish green coloured calli for which it was necessary to subculture after every 20 days as the calli growth decelerated. Sorbitol showed the lowest trend. The trend in the callus in the maltose and sorbitol fortified medium showed a gradual darkening of the callus which became intense and turned into dark brown probably due to the production and oxidation of phenolic compounds released by the tissue finally leading to the cessation of growth (Fig.1a-f; Table 2).

These results are comparable with those showing that glucose produce green and voluminous callus compared to other sugars [31]. The beneficial effect of glucose on callogenesis has also been mentioned in many plants [4], [11], [12], [13], [26]. Glucose is indeed the assimilated form of sugars by plant cells and the most important source of energy production [21]. 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 assimilable by plant cells. There was probably a competition between these two sugars for their assimilation by the cells that makes sucrose less active and consequently less auspicious to callogenesis in *C.anguria*. The results of the present study show that maltose and sorbitol have no beneficial effect on callogenesis, because these two sugars are in non-assimilable forms by plant cells. We found that glucose medium inhibited browning in agreement with works of some researchers [25]. Glucose resulted in less browning and better callus proliferation. Consequently, glucose was the sugar which induced the better response to callogenesis in *C.anguria*.

The results in the study indicate that there were tremendous variations in callus initiation under different concentrations of glucose. Several types of callus were distinguishable based on the physical appearance under different levels of glucose (Table 3). 1 and 2% of glucose in the medium brought an identical trend in callus type with nearly 10 and 15% of the explants producing green and compact calli. 30% of the cultures with 3% of glucose gave green compact callus with no necrosis. However, 40% of the cultures responded when the medium supplemented with 4% glucose supported the growth of green compact callus. This glucose level seems to have provided an adequate osmotic pressure that would permit absorption of mineral nutrients presents in medium which according to several authors are essential to cells growth [3], [20]. When the amount of glucose supplemented in the MS medium was increased to 5%, there was a decline in the response of the cultures to 25% and the callus was found to be green friable and browned. Nearly 15% of callus induction was seen when MS medium fortified with 6% of glucose was used in the medium. These morphologic observations are characteristic of embryogenic structure induction according several authors [19], [28].

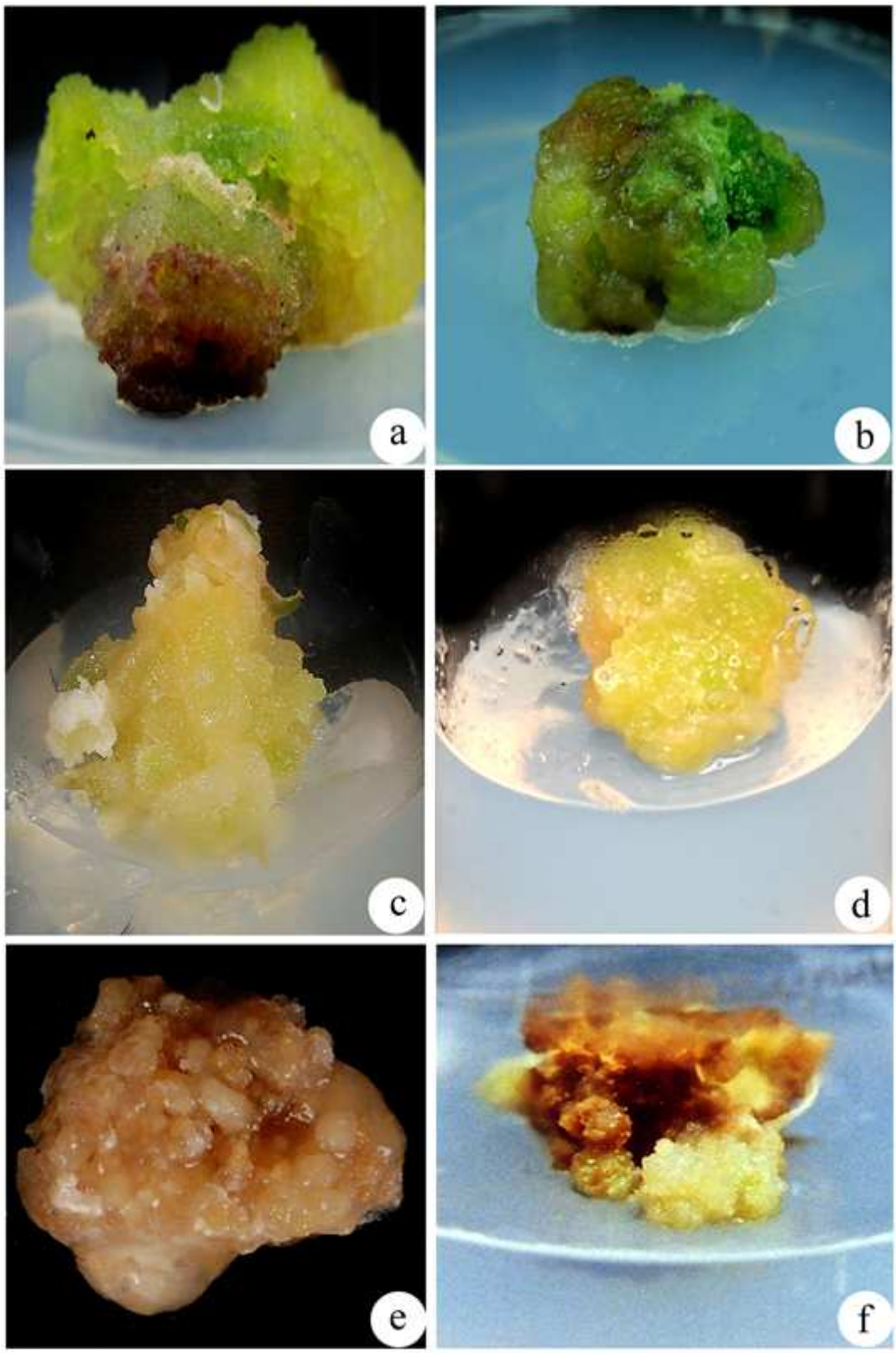

Fig 1. Callus induction in *C.anguria* under different types of sugars in MS medium (Murashige and Skoog medium, 1962) a. Glucose; b. Fructose; c. Sucrose%; d. Galactose; e. Maltose; f. Sorbitol

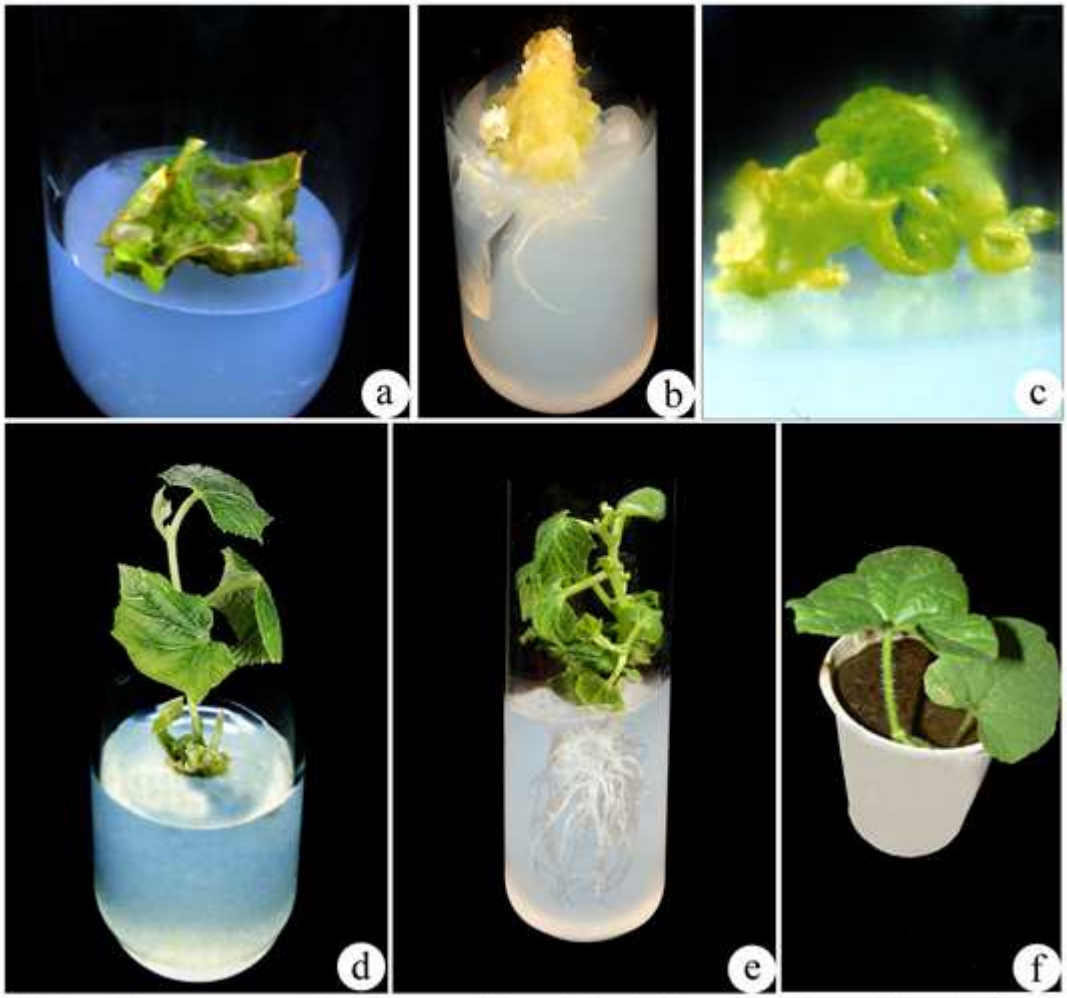

Fig 2. Plant regeneration from callus derived from *in vitro* cultured leaf segments of *C. anguria*. a. Leaf explants from *in vitro* raised seedlings; b. Callus induced MS + 2.0 mg L^{-1} 2,4-D & 2.0 mg L^{-1} NAA; c. Multiple shoots induced on MS + 3.0 mg L^{-1} BAP & 0.5 mg L^{-1} Kn; d. Shoot elongation on the same media; e. Rooting on MS + 3.0 mg L^{-1} NAA & 2.5 mg L^{-1} IBA; f. Acclimatized plant

Multiple shoot induction, proliferation

The callus obtained with optimum concentrations of glucose (Fig.2b) was subcultured on the media containing BAP in the range 1.0-5.0 mg L^{-1} and Kn at 0.5 mg L^{-1}.A variety of responses were observed which included enhanced shoot proliferation (Fig. 2c & 2d; Table 4). The best multiple shoot induction was observed after 20 days from the peripheral regions of the explants. BAP at its 3.0 mg L^{-1} concentration along with 0.5 mg L^{-1} Kn evoked best response with an average number of 8.70 shoots with a length of 6.32±1.2 cm. When BAP was decreased to 2.0 mg L^{-1} along with 0.5 mg L^{-1}, an average number of 7 shoots with a shoot length of 5.27±1.3 cm was produced. Incorporation of BAP at 1.0 mg L^{-1} along with Kn initiated bud proliferation but the shoots remained stunted. Higher concentrations of BAP brought a decrease in both the number of shoots and the shoot length. Shoots after their initial proliferation on medium containing 3.0 mg L^{-1} BAP and 0.5 mg L^{-1} Kn were sub-cultured on the same fresh medium after every 21 days to initiate shoot elongation and proliferation of multiple shoots.

The results are in complete agreement with an earlier study in which BAP was responsible for shoot induction in *Ctenolepis garcinii* when 2.0 mg L^{-1} BAP was used in MS medium [1]. In another study, maximum number of indirect (callus interspersed) regeneration of multiple shoots (9.0±0.5 shoots per explant) from leaf explants on MS

medium enriched with 2.5 μM BA alone in *Momordica cymbalaria* [18]. Similarly, the maximum shoot regeneration through the calli obtained from hypocotyl and proximal explants were obtained in *Cucurbita pepo* in the medium that included 0.5 mg L^{-1} BAP and 1.0 mg L^{-1} Kn [5]. The synergistic effect of kinetin and BAP brings about effective shoot induction from the callus. The present study has attempted to bring the same effect.

Rooting and Acclimatization

Full or half strength MS medium without any PGR did not support the rooting of regenerated shoots. However, shoots were capable to induce root when cultured on medium containing auxins. *In vitro* regenerated shoots produced roots when they were cultured on MS medium fortified with NAA and IBA. Maximum rooting was given on MS medium with 3.0 mg L^{-1} NAA and 2.5 mg L^{-1} (Table 5; Fig. 2e).

Total nine rooted plantlets were transferred from *in vitro* to *in vivo* conditions and successfully acclimatized in pots containing sterilized garden soil and sand mixture (1:1) in small PVC cups (120 mL volume) under greenhouse conditions for 8 weeks. Survival rate was 75% under field conditions which was calculated after 4 weeks (Fig.2f). The plantlet grew successfully into normal plant under natural conditions.

CONCLUSION

In vitro propagation of West Indian gherkhin provides excellent advantages over traditional propagation, including a high multiplication rate, physiological uniformity, the availability of disease-free material all the year round, rapid dissemination of new plant materials throughout the world, uniformity of shoots, short harvest interval in comparison with conventional plants, and faster growth in the early growing stages compared to conventional materials. The protocol raised in the present attempt could be used for the massive *in vitro* production of plantlets from gherkhin. The manipulation of culture conditions using various combinations and concentrations of growth hormones and other adjuvants can provide a reproducible protocol and reduce the high costs of plant production through vegetative means. Consequently, it will meet the pharmaceutical requirements besides strengthening solidarity to the increasing concern to conserve biodiversity through preservation of germplasm of this medicinal shrub.

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