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CULTIVATION OF LILIES (*LILIUM REGALE*) FOR COMMERCIALIZATION IN PAKISTAN

K. SAIFULLAH[1], N. SHEEBA[1], R. MARIAM[1], K. NAHEED[1], N. ASMA[1] AND S. BUSHRA[2]

[1]Plant Tissue Culture and Biotechnology Division. International Centre for Chemical & Biological Sciences, H.E.J. Research Institute, University of Karachi 75270, Pakistan
[2]Shaheed Zulfikar Ali Bhutto Institute of Science and Technology, (SZABIST), Clifton, Karachi, Pakistan

Abstract

The current study envisages producing *Lilium regale* plants on a large scale, employing cost-effective *in-vitro* protocols. The effect of various concentrations and types of plant growth regulators were observed to optimize the media for large scale propagation. Initiated buds were cultured on MS medium supplemented with BAP (0.5-4.0 mgL^{-1}); IBA (0.5 and 1.0 mgL^{-1}) and IAA (1.0 mgL^{-1}). A rich solid mass of white-green callus was produced. Greatest number and length of shoots were produced in a medium containing BAP (2.0 mgL^{-1}), IBA (1.0 mgL^{-1}), IAA (1.0 mgL^{-1}), Peptone (1.0 mgL^{-1}) and sugar (60 gL^{-1}). Rooting started on media containing half the concentration of MS salts, supplemented with NAA (1.0 mgL^{-1}) and activated charcoal (2 mgL^{-1}). Three weeks after a rich mass of roots were obtained, the plants were transplanted individually in small clay pots and kept in close tunnels where the relative humidity was approximately 40%. Four weeks after acclimatization, the plantlets were shifted into green houses.

Introduction

In recent years, propagation of numerous ornamental plants by tissue culture has become an accepted commercial practice. Current production techniques for *In vitro* propagation of plants have allowed for strong and continued growth within the micro-propagation industry. *Lilium* is one of the leading cut flowers all over the world (Kumar, 2007). It ranks seventh among the cut flowers in the world in terms of auction turnover, and is a very popular pot plant as well. There has been a big market demand for exotic cultivars of *Lilium regale* in Pakistan due to their majestic long slender perfumed and showy flowers, and these are being imported in ever increasing quantities. Parts of Pakistan that have a cool temperate winter have the potential of growing this fabulous exotic plant on a large scale. Introduction of this plant will undoubtedly play a significant role in offering a new dimensions to the existing horticulture industry which thus far operates on very traditional lines.

According to Van Aartrijk *et al*., (1990), the main constraints in conventional propagation of lilies include the inadequate availability of healthy, disease-free planting material, and slow multiplication rates. The conventional method of propagating lilies involves scaling yields at most three to five bulbs from each scale, depending on the bulb-scale size and species/ variety. Thus scaling a bulb yields somewhere between 50 and 100 bulblets, far too few to meet the present demand for planting material (Varshney, 2000).

One of the best and most prolific vegetative propagation method for lilies is *in-vitro* scale culture (Jeong, 1996; Varshney *et al*., 2001; Bahr & Compton, 2004). *In vitro* adventitious bud regeneration from scales of *Lilium* depends on factors such as concentrations of plant growth regulators employed, (Jeong, 1996; Varshney *et al*., 2000), concentrations of sucrose (Jeong, 1996; Varshney *et al*., 2000; Kumar *et al*.,

2005), light treatment (Varshney *et al*., 2000; Lian *et al*., 2002b; Kumar *et al*., 2005), scale position or kind of explant (Jeong, 1996; Varshney *et al*., 2000). The current study reports developing a micropropagation of regime for *L. regale* in which regeneration, followed by production of adventitious shoots on a large scale was optimized, and it is envisaged to yield showy, attractive flowers. The use of tissue cultured plant material can rationalize lilium cultures because direct planting into pots without special manipulation for a large harvest of bulbs is possible. Therefore micropropagation by means of *in-vitro* techniques is of great interest in order to speed up the propagation rate and to reduce the need for bulblets. The objective of this work was to define an efficient protocol for the micropropagation of lilium, cv. Regale, by the manipulation of Benzyl-3-amino purine (BAP) concentrations, type of culture medium and sectioning of the axillary buds used as explants, aiming at the maximization of the multiplication rate in order to make this a feasible mode of propagation.

Methodology

The explants employed were healthy, juvenile bulbs of *Lilium regale*, which were thoroughly washed under running tap water for 30-40 minutes after removal of outer scale covering. The explants were then soaked in a 250 ml sterile jar containing sterile distilled water with 5-6 drops of Tween 20. The jar was placed on a gyratory shaker for 15 minutes. The explants were soaked in a 0.2% solution of Topsin-M® fungicide, for 10 minutes, followed by four to five rinses with clean water. The explants were given a quick dip in 70% ethanol for 5 seconds followed by placing in a 2.5% solution of Sodium hypochlorite, (commercial bleach containing 4% active chlorine) containing 3 drops of Tween 20 on continuous but very gentle stirring on a gyratory shaker for 20 minutes. This was followed by three consecutive rinses in sterile distilled water for five minutes each to remove traces of the Sodium hypochlorite solution. All the sterilization work was done under laminar flow cabinets in sterile environment. Under aseptic conditions, most of the external scaly tissue was eliminated and explants cleaned and were individually introduced into 250ml glass jars containing 35ml of MS medium (Murashige & Skoog, 1962) supplemented with 30.0gL^{-1}sucrose, 5.0gL^{-1} gelrite and 0.5 mgL^{-1}BAP. The cultures were maintained in the growth rooms at 27ºC +-2ºC and a 16 h photoperiod (40 mmol. M-2s^{-1}). To optimise the best conditions for the proliferation of shoots, different treatments were defined, varying the type of medium (liquid or solid MS medium), the concentrations of BAP (0.0, 0.5, 1.0, or 2.0 mgL^{-1}) and varying the concentrations of IBA (0.5 or 1.0 mgL^{-1}). The shoot multiplication medium was supplemented with IAA (1.0 mgL^{-1}), Peptone (2.0 mgL^{-1}) and sucrose (60 mgL^{-1}). In another series of experiments, surgical cotton was employed as a supporting matrix for the plants in lieu of gelrite, this media type was denoted as 'liquid' medium. The experiment was laid out in a complete randomized design (2x10x4) with 2 types of media (solid, and cotton support media), 10 concentrations of media (coded M 1-M 10, as seen in Table 1) and with 4 replications each. During the proliferation stage, five subcultures were done at intervals of four weeks, to the same proliferation medium, and the cultures were maintained in the growth room under the same conditions of illumination and temperature used during the introduction stage. After the final cultivation cycle, the shoots were individualized and the number of shoots per initial explant in each treatment were counted. Callus formed if any, were cut off from the shoot using a sterile scalpel before transfer. The callus was then sub cultured onto media having the previous media composition (s), for further shoot production. The shoots were transferred onto fresh media with the cut ends towards the media. Data was recorded by counting the number and length of new shoots and roots per

plant. Subsequently, the shoot proliferation rates were compared on solid and liquid medium (static condition). Shoots were transferred to MS medium with half the concentrations in order to induce rooting, the plants were subjected to IBA and NAA at concentrations of 0.25, 0.50, 1.00, 2.00 mgL^{-1}, in 1/2MS each with 6 replications. Average number of roots and increase in root length (mm) were measured after a period of 7 days on each jar for a successive period of 6 weeks. When a considerable network of adventitious roots had been established the plants were transferred to green house for acclimatization. Plantlets with roots between 20-40 mm in length were randomly selected for transferring into green house. Plantlets were removed from culture, and the roots were gently washed in luke warm distilled water to remove any residual gel or medium, followed by a wash in 0.1% fungicide, Topsin-M. These plants were placed into 6 inch clay pots containing the following media: only sand, only peat moss, sand + farm yard manure (FYM) (50:50), sand + peat moss (50:50), FYM + peat moss (50:50), FYM + peat moss + sand in equal parts. The pots were placed in tunnels where the humidity was approximately varied from 30%- 80%. Data was recorded to determine which type of medium was optimum for acclimatisation. Over 4 weeks, relative humidity was slowly decreased by gradually shifting the plants into semi-shade areas. All experiments were of completely randomized design and repeated at least twice. Each treatment consisted of 80 explants (four explants per 250 mm pot). The number of newly emerged shoots and roots were subjected to square root transformation $(x + 1.0)$ ½ prior to analysis. The number and lengths of both roots and shoots were also presented as mean values with a standard error. Data was analyzed employing SPSS version 4.0 (Scientific Enterprises, USA).

Results and Discussion

The effect of a number of cytokinin and auxin concentrations in the growth media on explants was investigated. The individualized shoots were observed to produce lateral shoots simultaneously increasing in length over the 4 week passage (Fig. 1). Profuse adventitious shooting was observed in medium containing a combination of BAP at 2.0 $mgL^{-1}$and IBA at 1.0 mgL^{-1} (Fig. 1(e) and (f)) Maximum increase in shoot number was 8.40 with a standard error of ± 0.45 in the solid phase media, where gel was used as a supporting matrix (Table 1). Likewise, the maximum length of shoots was also obtained in the medium having the same combination and concentration of plant growth regulators. Greatest increase in shoot length obtained was 78 mm with a standard error of 1.01. The clumps of shoots were further dissected laterally, and transferred onto fresh media, in which they were observed to increase in size i.e. a further increment in the number of shoots was observed. Trimming of older leaves of shoots prior to subculture promoted axillary shoot formation most probably due to the suppression of apical dominance. The clumps of shoots were observed to produce a hard undifferentiated mass of white green cells, which when cut off from the 'callus' and transferred to fresh medium, were found to result in massive regeneration of new shoots. These tiny shoots, when transferred to fresh medium continued to produce adventitious shoots, and the callus continued increasing in size, simultaneously yielding fresh shoots. Therefore, a continued cyclic production of shoots was possible, leading to continuous cultures and generation of large number of shoots. A two- fold increase in multiplication was observed in the second subculture, followed by three- to four- increase in the subsequent subcultures. Based on these observations, the explants cultured for 12 weeks on the same medium, had attained the stage of vigorous proliferation, consisting of 8 to 10 shoots, and were divided into smaller clumps.

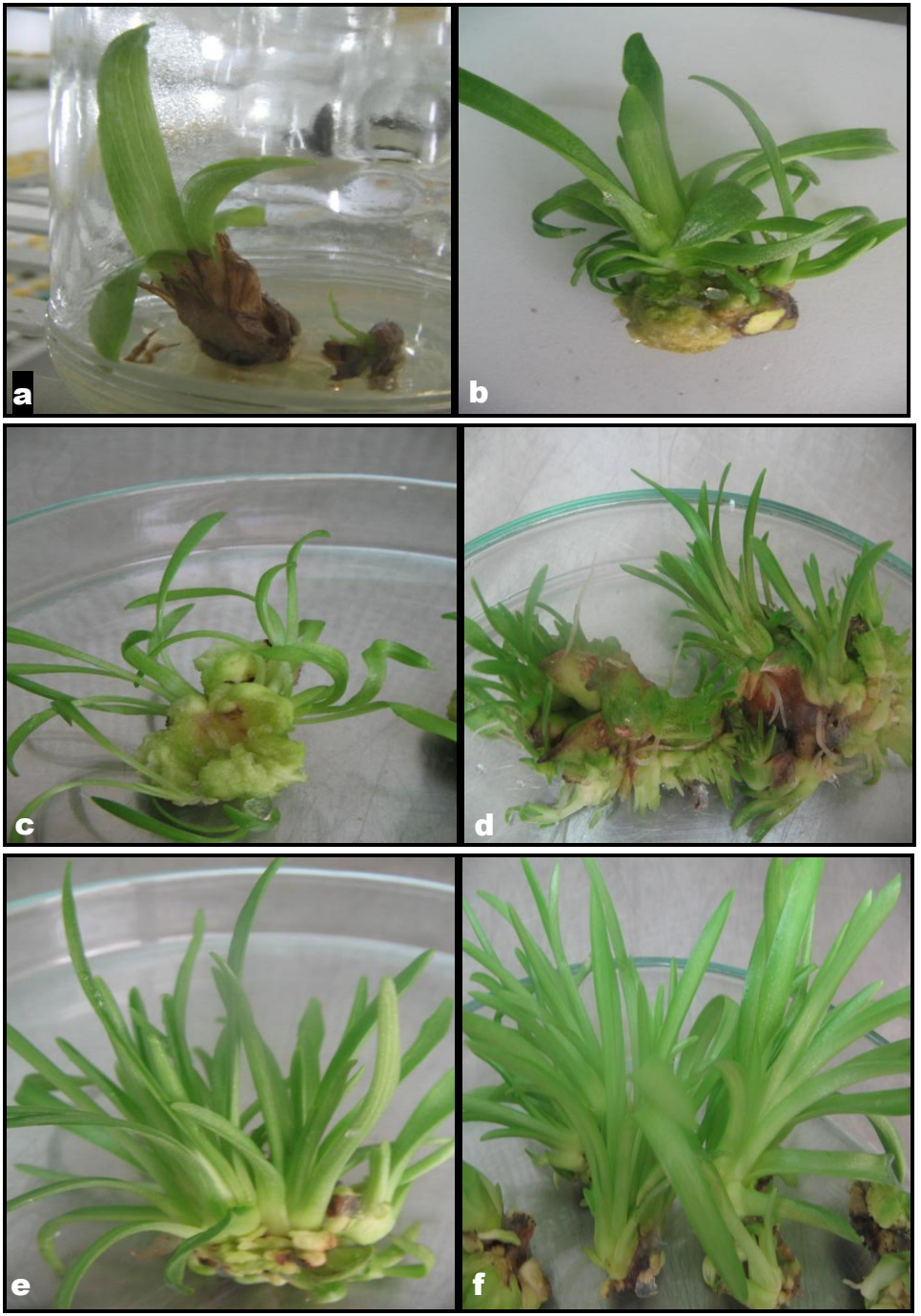

Fig. 1.(a) and (b) Initiation of bulbs on medium containing BAP at concentrations of 0.5 mgL^{-1}; (c) and (d) regeneration of shoots; (e) and (f) profuse multiplication and elongation of shoots on medium containing BAP (2mgL^{-1}), IBA (1mgL^{-1}), IAA (1mgL^{-1}) and Peptone (2 mgL^{-1}).

Each clump consisting of 3 to 4 shoots was further subcultured on the same (fresh) medium, where it was observed that the dwarf shoots recovered to normal growth with more number of axillary shoots arising from the base. At the same time, 2 to 3 vigorously growing shoots from each clump elongated with expanded leaves by 4 weeks. Such shoots had healthy, root initials. The elongated shoots (4 cm to 5 cm long) were excised and cultured separately in the same medium in order to encourage the formation of long shoots, broad leaves and basal roots. The regeneration potential was observed to be best in the medium containing BAP at 2.0 $mgL^{-1}$and IBA at concentrations of 1.0 mgL^{-1}, coupled with a combination with IAA at 1.0 mgL^{-1}, Peptone at 2.0 $mgL^{-1}$and sugar at 60 gL^{-1}. When a comparison was made against gel supporting media and cotton support media, a clear picture was drawn exhibiting that shoots in gelled media showed better results in terms of number and length of shoots, and also in terms of regeneration potential.

For root induction, when the shoots of considerable height (more than 3 inches) were transferred onto a medium containing half the concentrations of MS salts, they were observed to have a natural tendency to produce roots (Fig. 2a and 2b). The frequency of root induction and the rate of plantlet growth were slow when shoots were inoculated for rooting (liquid) under semi-submerged conditions. The basal tuft of rooting was observed in more then 95% of the transferred shoots. The elongated shoots attained a height of 5-6 cm in about three weeks and were ready to be transplanted. When several concentrations of IBA were employed the greatest average number of roots was 5.84 ± 1.84 in a medium containing 2.0 mgL^{-1} IBA (Table 2). However, the length of roots obtained were too small and not considered suitable for transfer in greenhouses. However, when IBA was substituted by NAA, it was observed that at 1.0 mgL^{-1}NAA, the percentage of rooting was 98%, the average number of roots was 10.38 ± 1.28 with a length of approximately 6 cm, which also required a considerable time of approximately 15 days for root induction.

Plantlets grown for 3-4 weeks *In vitro* were removed and thoroughly washed under running tap water, and soaked in fungicide, Topsin-M® (0.1 %) for a few minutes and planted in 6 inch pots. This was followed by covering with transparent plastic for increased humidity. Transfer of plantlets with sterile healthy roots showed 100% survival in greenhouses in 6 inch clay pots. The established plants were gradually exposed to the open environment where they showed rapid growth.

On conducting a series of experiments, it was found that an ideal media for acclimatization was one which contained only peat moss (Fig. 1). Superficial planting in well- aerated soil types led to good and reproducible results in terms of plant vigor. The ideal temperature for acclimatizing the microcuttings was observed to be between 15-18ºC. An N-P-K spray (10:10:15) was applied once a week, and the dosage was gradually decreased to once every 10 days, and the plants were lightly watered once every two days. All the plants subjected to acclimatization are surviving cent per cent in the green house (Fig. 3a-c). It was further observed that an optimum humidity of 30% was ideal for acclimatizing the young lily plantlets, as most probably the low levels of humidity prevent roots from rotting, eventually resulting in healthy plant growth. Low levels of humidity were important to prevent parasitic diseases to which Lilium plants are highly susceptible to.

The present study elucidates an *In vitro* multiplication of *Lilium regale* and also indicates that *Liliums* can be grown successfully in solid medium. No evidence of vitrification has been found during a 10-month period of continuous culture. Use of liquid medium was also successful but with a low and unstable multiplication rate of shoots. However, other reports have suggested that a cotton support medium results in a higher regeneration potential of axillary shoots, as has been observed in banana plants (Khan *et al*., 2001).

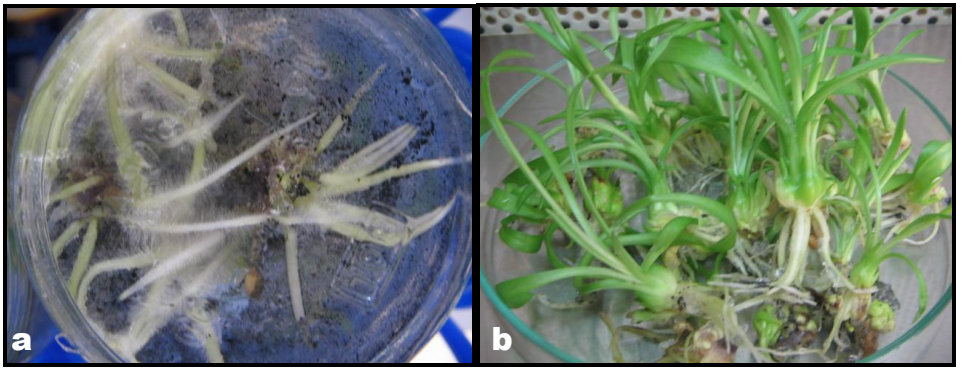

Fig. 2. Profuse rooting observed in micropropagated lilies (a) star pattern of rooting observed *In vitro* (b) Plantlets with healthy and roots developed on medium containing half concentration of MS salts, coupled with NAA at concentrations of 1 mg L-1 ready to be transferred in the fields.

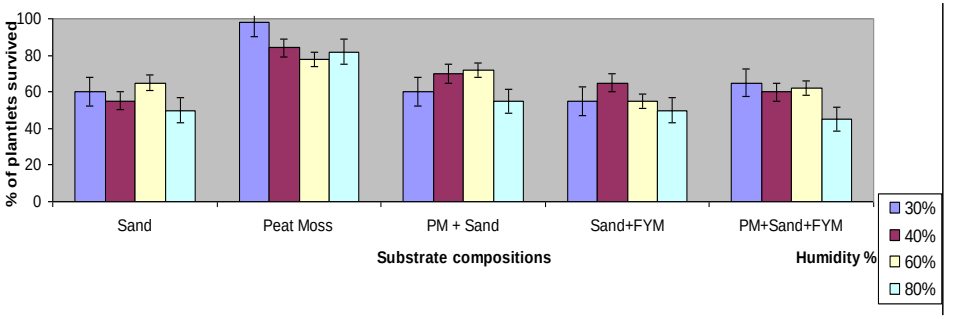

Fig. 1. Effect of different substrates and humidity levels on acclimatized *In vitro* lily plantlets.

Fig. 3. Plantlets being acclimatized in green houses (a) high humidity levels of 80% are maintained by covering with plastic sheets; (b) healthy plantlet ready to be shifted under direct sunlight; (c) Experimental 'plot' under observation.

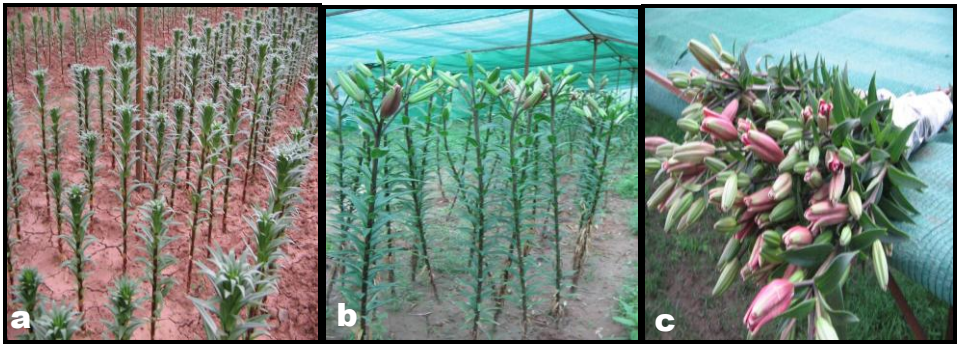

Fig. 4. (a) Plantlets being cultivated in fields; (b) healthy plantlets sprouting buds; (c) A freshly plucked bouquet of *Lilium regale*.

The method of callus culture followed by regeneration was preferred to shoot tip culture for micropropagation as it maximizes the rate of shoot multiplication, thus making this a very feasible protocol, which can be conveniently employed. The results of this study suggests that instead of 3 different medium, one media under same growth conditions was sufficient for the successful commercial micropropagation. This is in contrast to the findings of Debergh, (1990) and Maene (1988), who employed a high light intensity of 100 µM (sec.m^2) for shoot elongation. Utilization of the procedure described here offers simple medium and culture procedure for multiplication of lilies in a shorter duration and with a continued cyclic production of plantlets on a large scale to meet the ever rising market demands (Fig. 4a, b, c).

Commercial *In vitro* propagation schemes for lilies have been reported on adventitious bulblet formation from scale tissue (Takayama & Misawa, 1983). Such schemes have been theoretically correct in being able to yield several million of plants from one mature bulb in a single year. However, it should be realized that successive transfers of bulb scales on identical environmental conditions may lead to declining regeneration responses, thus resulting in a variation of the physiological characteristics of these scales from repeated transfers. Several attempts have been made to multiply lily bulbs through tissue culture, however, although the bulblets raised showed a high multiplication rate, for commercial use they remained small in size (Niimi, 1995, Kumar *et al*., 2001). Recent reports suggest that commercial use of micropropagation of lily bulbs is being reduced due to the high production costs resulting primarily from high labour costs (Lian *et al*., 2003). Studies with direct field planting of bulblets produced *In vitro* have shown that small bulblets emerge slower and are less uniform (Lian *et al*., 2003); and some recent studies have reported that most micropropagation systems have produced bulblets with a median fresh weight of <2g. Successful tissue culture for rapid propagation of some species of the genus Lilium has been reported including *L. longiflorum* (Tanimoto & Matsubara, 1995; Arzate-Fernandez *et al*., 1997; Nhut, 1998; Nhut *et al*., 2001; Nhut *et al*., 2002; Bacchetta *et al*., 2003; Nhut, 2003), *L. japonicum* (Yamagishi, 1998), *L. speciosum* (Chang *et al*., 2000), *L. concolor* (Jeong, 1996), *L. nepalense* (Wawrosch *et al*., 2001), *Lilium oriental hybrid* (Lian *et al*., 2002a), *L. ledebourii* (Azadi, 2007).

Thus, this study is of significance as it by passes bulb production, thus reducing costs and eventually making the micropropagation regimes for mass production of lilies feasible, producing healthy flowers to satisfy consumer demands. *In vitro* techniques have become increasing important in the production of good quality and quantity of crops.

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