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High frequency multiple shoot regeneration from decapitated embryo axes of chickpea and establishment of plantlets in the open environment

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Abstract

Multiple shoot regeneration from the cut plumular ends of embryo axes of chickpea (*Cicer arietinum* L.) was evaluated on Murashige and Skoog medium having different concentrations of thidiazuron (TDZ) (0.1 to 10.0 mg dm^{-3}), 6-benzylaminopurine (BAP) (0.5 and 1.0 mg dm^{-3}), kinetin (0.5 and 1.0 mg dm^{-3}) or zeatin (2.0 and 4.0 mg dm^{-3}). TDZ (0.2 mg dm^{-3}) was found to be the most effective cytokinin as it produced multiple shoots in 100 % of the explants from genotypes C235, ICC5166, ICC12269, ICC4951, ICC11531, BG256 and a local cultivar. Shoots were elongated on growth regulator-free medium, and rooted on growth regulator-free medium containing 1/4 MS salts + full vitamins + 3 % sucrose. Plantlets formed were acclimatized for 12 - 15 d in MS medium with a gradual reduction in sucrose concentration and transferred into pots filled with soil and kept in the field; this resulted in more than 70 % survival. The plants developed normally and produced fertile flowers and set seeds. Low temperatures, maximum 19.0 °C, and minimum 8.2 °C, during the first 15 d of transfer favoured survival on transfer to pots.

Additional key words: acclimatization, auxins, *Cicer arietinum*, cytokinins, low-temperature, thidiazuron.

Introduction

Chickpea (*Cicer arietinum* L.), an important grain legume crop, exhibits low frequency of regeneration of shoots and somatic embryos, and poor survival of *in vitro*-raised plantlets upon their transfer to soil. Somatic embryogenesis has been reported from leaflets (Barna and Wakhlu 1993, Kumar *et al.* 1994) and immature cotyledons (Eapen and George 1994, Hita *et al.* 1997), while shoot regeneration has been reported from mature seeds (Polisetty *et al.* 1997), cotyledonary nodes, shoot-tips (Brandt and Hess 1994), decapitated embryo axes (Fontana *et al.* 1993, Kar *et al.* 1996) and immature cotyledons either directly (Srivastava *et al.* 2001) or through cotyledon like structures (CLS) (Shri and Davis 1992, Srivastava *et al.* 2001). Fontana *et al.* (1993) obtained one, occasionally more, shoots per explant in the case of 40 - 50 % responding decapitated embryonic axes (EA). However, survival of tissue culture-raised plantlets on transfer to soil has remained a major bottleneck; recently, a survival of 60 - 80 % was reported for plantlets transferred to vermiculite/soil and maintained under the controlled and sterile environments of the culture room (Polisetty *et al.* 1997). But it is not clear as to how many plants were transferred to pots and if the plants were transferred to the open environment.

This study was undertaken with the objective to develop a high frequency plantlet regeneration protocol using decapitated EA explants and to improve the survival of plantlets thus obtained, following their transfer to the open environment in pots filled with non-sterilized soil.

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Abbreviations: BAP - 6-benzylaminopurine; EA - embryonic axis; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; NAA - α-naphthalene acetic acid; TDZ - thidiazuron.
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Materials and methods

Six chickpea (*Cicer arietinum* L.) genotypes, C235, ICC5166, ICC12269, ICC4951, ICC11531, BG256 and a local cultivar, were tested for shoot regeneration; C235 was used for all the other experiments. Mature seeds were washed with 1 % *Cetrimide* solution (*ICI India*, Madras, India) for 5 min, rinsed for 30 s in 70 % ethanol, treated with 0.2 % mercuric chloride for 7 min and rinsed 5 - 6 times with sterilized distilled water. These seeds were soaked overnight in sterile distilled water, and their embryo axes (EAs) dissected out. Shoot and root apices of the EAs were removed and the remaining portion of the EAs, here referred to as decapitated EAs, were used as explants. Removal of ~1 mm of EA plumular tip produced the maximum number of shoots, while removal of more than 1 mm reduced the number of shoots per EA.

Murashige and Skoog (1962; MS) medium containing 3 % (m/v) sucrose was routinely used. For shoot multiplication, the medium was supplemented with 4.0 g dm^{-3} casein hydrolysate, since this reduced vitrification, improved the quality of shoots and promoted rooting; this medium is designated as MSM medium. Different concentrations and combinations of growth regulators (GRs) were added to the medium as per need of the experiment. Medium pH was adjusted to 5.8, it was solidified with 0.8 % purified agar (*Sigma*, St. Louis, USA) and distributed in 100 cm^3 conical flasks (30 cm^3 per flask) before autoclaving at 1.05 kg cm^{-2} for 20 min at 121 °C.

Six decapitated EAs were placed vertically in each flask, and the cultures were incubated at 18 or 24 °C under 16-h photoperiod and irradiance of 30 µmol m^{-2} s^{-1}. After 7 d of culture on a cytokinin-supplemented MSM medium, EA explants were transferred to GR-free MSM medium for shoot bud development and shoot elongation; the explants were subcultured every 2 weeks onto fresh medium of the same composition. After about 2 weeks of the first transfer, individual shoots of 2 to 3 cm length were excised and used for rooting. The mother explants along with the remaining smaller shoots were further subcultured to obtain further supplies of elongated shoots.

Healthy shoots of 2 to 3 cm were rooted on medium containing 1/4 strength MS salts + full vitamins + 3 % sucrose + 1.0 g dm^{-3} casein hydrolysate. We had evaluated the effects of 2×, 1×, 1/2× and 1/4× MS vitamins on rooting; of these, full strength vitamins was the optimum. Addition of 1.0 g dm^{-3} casein hydrolysate to the MS medium improved the quality of roots, but higher concentrations were inhibitory. The rooting medium was supplemented with 0.0, 0.1, 0.2, 0.5, 0.75 and 1.0 mg dm^{-3} IAA or IBA or the shoots were given a pulse treatment with 25 or 50 mg dm^{-3} IBA for 5 min, and then cultured on GR-free rooting medium.

Initially, we attempted to acclimatize plantlets following Barna and Wakhlu (1993) and Polisetty *et al.* (1996), but the rate of plantlet survival on transfer to open environment was very low (~10 %). Shoots with well-formed roots were acclimatized in liquid medium containing 1/4 - strength MS salts. In control, 30.0 g dm^{-3} sucrose was added to the medium throughout the period of hardening. In one treatment, no sucrose was added to the medium during hardening. In both these cases, plantlets were transferred to fresh medium every 3 d. In the second treatment, sucrose concentration was gradually reduced in the following series: 30.0→20.0→10.0→5.0→0.0 g dm^{-3}, the plantlets being kept for 3 d on each sucrose concentration. After 15 d, all the plantlets were transferred to 1/4 strength MS salts liquid medium. The cotton plugs of culture tubes were loosened for 3 - 4 d, followed by their removal for gradually increasing periods so that in another 6 - 7 d the plugs were completely removed.

Acclimatized plantlets were transferred to plastic pots containing autoclaved 1:1 mixture of *Soilrite* (*Kel Perlite*, Vishwasnagar, Karanataka, India) and garden soil, and covered with polyethylene bags for 1 - 2 d. These plantlets were kept under culture room conditions for about 7 d, before being gradually exposed to sunlight for the next two weeks. The hardened plantlets were then transferred into big cement pots kept in the open environment and filled with fresh garden soil. These plantlets were shaded for 10 d by covering them with newspaper between 11:30 and 15:30.

At the end of hardening period, half the number of plantlets in each treatment was randomly selected for dry mass determination, while the remaining half of the plantlets were used for chlorophyll determination. Shoots and roots of each plantlet were separated and oven-dried at 70 °C for 24 hr and their dry mass was recorded. Chlorophyll was extracted by grinding fresh leaves in 80 % acetone using mortar and pestle and quantified spectrophotometrically according to Arnon (1949).

The degree of senescence was scored on an arbitrary scale, where zero represented lack of senescence, and 4 represented plant death. Data were subjected to analysis of variance according to randomized block design or nested design, and means were compared using Duncan's new multiple range test (Gomez and Gomez 1984).

Results and discussion

Shoot regeneration: Shoot regeneration from decapitated EA explants of C235 was evaluated on MS medium supplemented with one of the cytokinins: TDZ, kinetin, BAP or zeatin. TDZ was used at concentrations

0.0, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0 and 10.0 mg dm^{-3} either alone or in combination with 0.0, 0.1 and 0.5 mg dm^{-3} IBA or NAA, while kinetin or BAP were used alone at concentrations 0.5 and 1.0 mg dm^{-3}; zeatin was used at concentrations 2.0 and 4.0 mg dm^{-3}. In all the treatments, 100 % of the EAs produced shoots. TDZ alone induced 5.8 (at 0.1 mg dm^{-3}) to 10.1 (at 5.0 mg dm^{-3}) shoots per explant (Fig. 1), while BAP, kinetin and zeatin produced 1.4 to 2.4 shoots per explant. This is in conformity to the earlier report of multiple shoots derived from mature seed cultures of chickpea, lentil and pea (Malik and Saxena 1992), and from peanut (Radhakrishnan *et al.* 2000). EAs

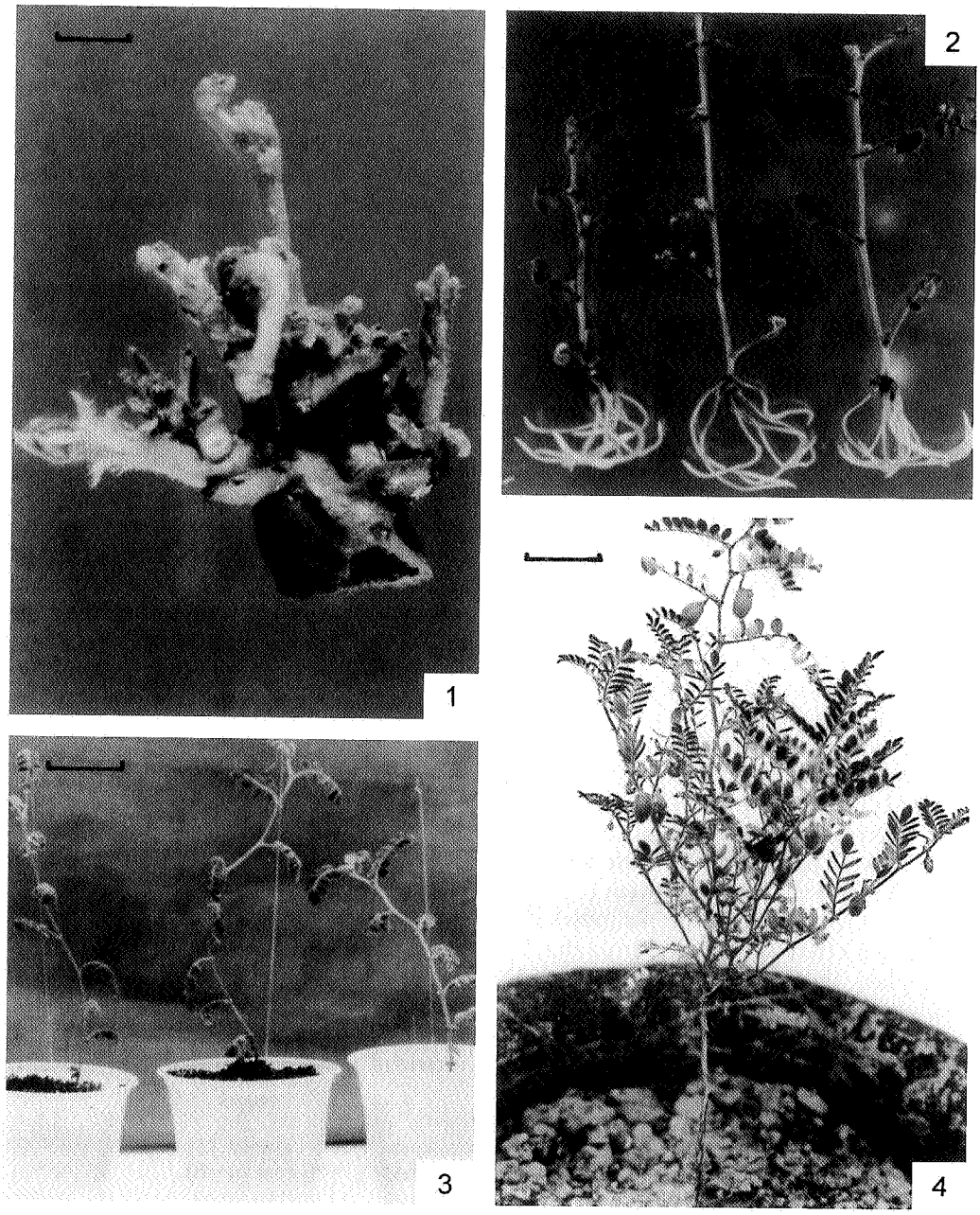

Fig. 1. Multiple shoot regeneration from decapitated EA of chickpea cv. C235 cultured on MS medium with 0.2 mg dm^{-3} TDZ (*bar* = 2.0 mm).
Fig. 2. Regeneration of roots from elongated shoots of chickpea cv. C235 on 1/4 MS salts + full vitamins + 0.1 mg dm^{-3} IBA (*bar* = 1.0 cm).
Fig. 3. *In vitro* regenerated plantlets of chickpea transferred into plastic cups kept in the culture room (*bar* = 2.0 cm).
Fig. 4. Pod formation in a chickpea plant after it was transferred from plastic cup into a pot kept in the open environment (*bar* = 5.0 cm).

cultured on TDZ containing medium produced shoots for 4 - 6 subcultures on GR-free MS medium; a total of 23 - 30 shoots were obtained from each EA explant after these sub-cultures, which was not found with other cytokinins. Kanyand *et al.* (1994) have also reported several cycles of shoot regeneration in *Arachis hypogaea* in response to continuous exposure to 0.5 - 30 mg dm^{-3} TDZ. Although the higher (0.5 to 10.0 mg dm^{-3}) concentrations of TDZ produced higher number (9 - 10) of shoots per explant, these shoots were distorted or fasciated and did not elongate normally. Addition of IBA or NAA in combination with TDZ significantly suppressed the number of shoots per EA. This could be because TDZ itself modulates endogenous level of auxin and substitutes for combined cytokinin and auxin requirements (Hutchinson *et al.* 1996). Therefore, TDZ at 0.2 mg dm^{-3} was considered optimum for multiple shoot production from C235 EAs. EAs were cultured on 0.2 mg dm^{-3} TDZ for 3, 5, and 7 d before being transferred to GR-free medium. The maximum number (7.3) of shoots was obtained on 7-d exposure, followed by that on 5-d and 3-d (5.6 and 5.8 shoots per EA, respectively) exposures; however, the differences among these values were nonsignificant. An exposure longer than 7 d to TDZ resulted in short and compact shoots, which were difficult to elongate and root. These observations are in conformity with those of Lu (1993). Thus the brief exposure (7 d) of EA explants to a low concentration of TDZ, followed by transfer to GR-free medium appears to be economical for use, *e.g.* in genetic transformation.

Six genotypes of chickpea and a local cultivar were evaluated on MSM + 0.2 mg dm^{-3} TDZ medium; multiple shoots regenerated from 100 % EAs. The highest number of shoots per EA (9.7) was obtained in ICC4951, while ICC11531 showed the lowest response (3.8 shoots per EA). The local cultivar and genotypes ICC5166, ICC12269, BG256 and C235 showed intermediate response (6.6 to 5.6 shoots per EA). Clearly, this regeneration mode is not genotype-specific although genotype had a marked effect on the number of shoots regenerated per explant. In contrast, shoot regeneration from other explants and somatic embryogenesis are reported to be highly genotype-specific (Eapen and George 1994).

Rooting: Initially the effect of 0, 1, 2 and 3 % sucrose was tested on rooting; 3 % sucrose produced healthiest roots without any detrimental effect on the frequency of rooted shoots. 1/4 MS salts in the rooting medium was superior to 1/2 or full MS salts, more possibly due to a reduced nitrogen content than a reduced osmotic potential (Hyndman *et al.* 1982). Similarly, rooting in *Solanum nigrum* shoots was the best on 1/2 MS salts + 1 mg dm^{-3} IBA (Hassanein and Soltan 2000). About 76.7 % of the shoots rooted on GR-free rooting medium. Pulse treatment for 5 min with 25 or 50 mg dm^{-3} IBA resulted in rooting of 100 % of the shoots; it also produced the highest number of (6.3 and 6.1) roots per shoot and the longest (2.6 and 2.3 cm) roots. But pulse treatment is time consuming and shoots got desiccated during the treatment; therefore, this procedure was not used further. However, addition of 0.1 to 1.0 mg dm^{-3} IBA in the rooting medium did not improve the frequency of rooted shoots over that in the GR-free medium, and tended to suppress root growth. Further, the presence of IAA or IBA at 0.5 mg dm^{-3} and higher concentrations in the medium induced callusing at the base of shoots; the roots ordinarily regenerated from this callus and were weak. IBA at 0.1 mg dm^{-3} supported the best root formation (Fig. 2), but GR-free 1/4 MS salts medium supported almost as good root formation; therefore, shoots were regularly rooted on the GR-free medium. In case of *Anacardium occidentale*, a 5-d induction period was more suitable than continuous IBA treatment for rooting of the shoots (Boggetti *et al.* 2001).

Effect of temperature: Chickpea tissue culture seems to be sensitive to temperature. A temperature of 24 °C adversely affected the number as well as the quality of shoots produced from EAs (Table 1). The shoots were thin and weak, and they senesced early in cultures kept at 24 °C. In addition, the mother explants released considerable amount of phenolic compounds and they had to be frequently subcultured. In comparison, EA cultures incubated at 18 °C produced significantly more shoots per EA, the shoots were relatively healthier, appeared sturdier, and released much less phenolics in the medium. Fonnesbech and Fonnesbech (1979) reported increased cytokinin [N-benzyl-9-(2-tetrahydroperanyl)

Table 1. Effect of temperature on shoot and root regeneration in chickpea. Means followed by different letters are significantly different at $P = 0.05$ (* - number of shoots cultured, ** - number of roots [$shoot^{-1}$], *** - root length).

	Temperature [°C]	Number of EAs cultured	responded	Number of shoots [EA^{-1}]	Shoot height [cm]
Shoot regeneration	18	50	50	7.6b	0.8
	24	50	50	5.4a	0.9
Root regeneration	18	25	24*	4.2b**	1.9***
	24	25	18*	2.2a**	1.9***

adenine] requirement at higher temperatures in *Asparagus plumosus* shoot-tip cultures; at 17 °C, 0.2 mg dm^{-3} was optimum, while at 24 °C the optimum concentration was 2.0 mg dm^{-3}. Frequency of rooting as well as the number of roots per explant were also suppressed at 24 °C as compared to those at 18 °C (Table 1). In contrast, roots did not develop at 19 °C in case of *A. occidentale* shoots (Boggetti *et al.* 2001).

Temperature had a marked effect on the plantlets during hardening. Shoot and root dry masses were significantly higher at 18 °C than those at 24 °C, but chlorophyll content was comparable. There was more pronounced senescence at 24 °C than at 18 °C (Table 2). It appears that plants were stressed at 24 °C.

Table 2. Effect of temperature on shoot and root growth of chickpea plantlets during hardening. Means followed by different letters are significantly different at $P = 0.05$.

Temp. [°C]	Shoot d.m. [mg]	Root d.m. [mg]	Senescence [a.u.]	Chl [mg g^{-1}(f.m.)]
18	30.8b	16.7b	1.7a	0.85
24	16.9a	8.7a	2.0b	1.04

Plantlet establishment and survival in the outdoor environment depended mainly on the ambient temperature during the first 15 d after transplanting. When the minimum and maximum temperatures during this period ranged between 6.2 and 10.2 °C, and between 14 and 24.0 °C, respectively, ~73 % of the plantlets survived. But when the minimum and maximum temperatures were higher, plantlet survival declined to ~33 %. It was further reduced to only ~17 % when the minimum and the maximum temperatures ranged between 9.8 and 17.3 °C, and between 23.0 and 30.2 °C, respectively (Table 3).

Table 3. Effect of sucrose during acclimatization (3 % or gradual decrease from 3 to 0 %) and of temperature during first 15 d of establishment in soil on survival of chickpea plantlets in the field; 40 plantlets were transferred in every treatment.

Sucrose	Temperature [°C] min.	max.	Surviving plants number	[%]
3→0	8.2	20.2	29	72.5
3	8.2	20.2	16	40.0
3→0	10.5	24.3	13	32.5
3	10.5	24.3	5	12.5
3→0	12.4	25.1	7	17.5
3	12.4	25.1	7	17.5

Acclimatization: *In vitro* produced plantlets when transferred directly from the rooting medium to sterilized *Soilrite* succumbed to fungal attack within 10 - 14 d. Therefore, we tried to acclimatize these plantlets by a gradual reduction in sucrose concentration during hardening at 18 and 24 °C. Shoot and root dry masses were the lowest for plants hardened on 0 % sucrose. Shoot and root dry masses were comparable on 3 % sucrose with those on gradually reduced sucrose concentration, except for shoot dry mass at 18 °C and root dry mass at 24 °C (Table 4). This is similar to the previous reports by Koroch *et al.* (1997) in *Hedeoma multiflorum*.

Senescence of plantlets was the lowest on gradually reduced sucrose concentration; the effect on senescence was more pronounced at 24 than at 18 °C. The amount of chlorophyll in plantlets hardened on 0 % sucrose and gradually decreased sucrose conetnt was significantly higher than that in plantlets hardened on 3 % sucrose. The reduced amount of chlorophyll in the presence of sucrose has been reported by Serret *et al.* (1996).

Acclimatization should enable *in vitro*-raised plantlets to change their mode of nutrition from heterotrophic to photoautotrophic, and to have enough nutrient reserve to support them until they have established themselves in the new environment. Therefore, any treatment during acclimatization that would increase the photoautotrophic capacity and provide good amount of stored nutrient may improve plantlet establishment.

In case of chickpea, gradual reduction in sucrose was superior to 3 % sucrose in terms of chlorophyll content and to both 0 and 3 % sucrose in terms of lower senescence and general plantlet health. Langford and Wainright (1987) reported beneficial effect of gradual sucrose reduction in rose.

When hardened plantlets were transferred to 400 cm^3 glass bottles or 200 cm^3 plastic cups half filled with *Soilrite* (Fig. 3), there was 90 - 95 % survival in the case of gradual reduction in sucrose. The plantlets hardened on 0 % sucrose were weaker and presented difficulty during transfer to *Soilrite*. Therefore, only plantlets hardened using the other two treatments were transferred to soil in the open environment. At lower temperatures hardening with gradual reduction in sucrose appeared to be markedly superior to that on 3 % sucrose. This superiority almost disappeared at the higher temperatures (Table 3). Thus it appears that survival of chickpea transferred in the open environment depends primarily on ambient temperature, particularly the maximum temperature range, which should be ~20 °C or less. At maximum temperatures of 24 °C or more, hardening procedure did not appear to have a noticeable effect on plantlet survival.

An efficient procedure for multiple shoot regeneration from cut plumular ends of EA explants, complete plantlet formation and hardening has been developed. When hardened plantlets were transferred in the open environment, ~73 % of them survived and produced fertile flowers and set seeds (Fig. 4).

Table 4. Effect of sucrose concentration and temperature during hardening of chickpea (genotype C235) plantlets. Means followed by different letters are significantly different at $P = 0.05$.

Sucrose	Temperature [°C]	Shoot dry mass [mg]	Root dry mass [mg]	Senescence [a.u.]	Chlorophyll [mg g^{-1}(f.m.)]
0	18	48.4a	11.9a	3.0c	1.00b
3→0	18	62.6b	18.1b	0.8a	0.96b
3	18	73.9c	20.1b	1.3b	0.60a
0	24	20.6a	6.5a	3.5c	1.11b
3→0	24	39.2b	11.0c	0.7a	1.15b
3	24	41.4b	8.0b	1.8b	0.87a

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