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SILVER NITRATE PROMOTES SHOOT DEVELOPMENT AND PLANT REGENERATION OF CHILE PEPPER (*CAPSICUM ANNUUM* L.) VIA ORGANOGENESIS

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Summary

Chile pepper (*Capsicum annuum* L.) plants were regenerated from cotyledon explants *in vitro* in four major stages: bud induction, bud enlargement, shoot elongation, and root development. Bud induction medium contained 0.5 mg/L (2.9 μ*M*) indole-3-acetic acid and 2 mg/L (8.9 μ*M*) N^6-benzyladenine. Bud enlargement occurred, and an occasional shoot appeared when medium with 2 mg/L (6 μ*M*) gibberellic acid, 2 mg/L (8.9 μ*M*) N^6-benzyladenine, and 5 mg/L (29.4 μ*M*) silver nitrate was used. Most shoots elongated after placement on a third medium without plant growth regulators or on fresh plates of bud enlargement medium. Incubations were for 2, 2, and 4 weeks, respectively, at 28.5° C and continuous light. Treatment with silver nitrate was necessary for multiple shoot production and elongation to occur in the third culture stage and was most effective when present in the second-stage medium but not in the bud induction medium. Sixteen to 26% of the shoots rooted in medium with 1 mg/L (5.4 μ*M*) 1-naphthaleneacetic acid after 1 month. Additional shoots transferred to a second rooting medium with 0.1 or 1.0 mg/L (0.54 or 5.4 μ*M*) 1-naphthaleneacetic acid developed roots, increasing the overall rooting efficiency to 70–72%. Most rooted shoots grew well and produced viable seeds when grown in the greenhouse. Other cytokinins tested for plant regeneration were zeatin and thidiazuron. Zeatin induced few shoots and fewer well-developed plants. Thidiazuron induced multiple shoots 4 months after culture began, but many were small and did not elongate further. Phytagar tissue culture grade proved superior to other agars tested, increasing bud induction frequency from 0–33% to 80–93% and eliminating explant hyperhydricity.

Key words: pepper; *Capsicum annuum*; silver nitrate; plant regeneration; shoot organogenesis.

Introduction

Peppers (*Capsicum annuum* L.) are an important vegetable crop worldwide. In Mexico and the southwestern U.S.A., chile peppers are a major economic crop. The cultivars grown, however, are susceptible to fungal pathogens that not only destroy the chiles but persist in the fields. One approach to the development of pathogen-resistant peppers is the use of genetic transformation techniques, which currently require efficient plant regeneration protocols. However, published protocols for peppers (Gunay and Rao, 1978; Fari and Czako, 1981; Agrawal et al., 1989; Arroyo and Revilla, 1991; Ebida and Hu, 1993) have not efficiently produced plants from *Capsicum annuum* cvs. Joe E. Parker and New Mexico 6–4 (Phillips and Hubstenberger, 1985), which are important chile peppers. Elongation of shoot buds appears to be the limiting step in pepper regeneration (Phillips and Hubstenberger, 1985). In a recent report, $AgNO_3$ was included in bud induction media, producing buds on hypocotyl explants that developed into shoots and eventually into well-developed pepper plants (Valera-Montero and Ochoa-Alejo, 1992). Silver nitrate originally was mentioned in an abstract reporting on factors affecting pepper regeneration (Jacobs and Stephens, 1990). Comparative studies without $AgNO_3$ were not included or mentioned, but these references indicated that $AgNO_3$, an ethylene inhibitor known to enhance shoot production in other plants (Purnhauser et al., 1987; Chi et al., 1990), might be beneficial for pepper regeneration.

Most pepper regeneration protocols induce shoot buds on explants with N^6-benzyladenine (BA) and indole-3-acetic acid (IAA), then transfer buds to rooting medium in which a few shoots elongate and root. Use of glucose in the bud induction medium as well as warm-temperature incubation (28.5° C) under continuous light are critical factors in pepper organogenesis (Phillips and Hubstenberger, 1985). Some investigators have used a second-stage medium with gibberellic acid (GA) to enhance shoot elongation (Harini and Lakshmi Sita, 1993). Despite several attempts with *Agrobacterium* to transform peppers with a shoot organogenesis protocol, there has been as yet only one unconfirmed report in the refereed literature demonstrating the recovery of stably transformed plants (Lee et al., 1993; Fari and Andrasfalvy, 1994).

The primary question addressed in this report is whether the presence of $AgNO_3$ during one or more developmental stages of organogenesis enhances the regeneration of plants from explants of *Capsicum annuum* cv. Joe E. Parker and specifically whether it can mitigate the bud elongation problem encountered by most pepper researchers. Our results confirmed that $AgNO_3$ was required for shoot organogenic regeneration and demonstrated that it was most effective when present in the second-stage medium but not the bud induction

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medium. In addition, a third culturing stage was needed to optimize shoot elongation which occurred over a prolonged period.

Materials and Methods

Explant preparation. Seeds of *Capsicum annuum* L. cvs. NM 6-4 and Joe E. Parker (developed from NM 6-4) were obtained from Enchanted Seeds, Anthony, New Mexico. Seeds were surface-sterilized in 95% ethanol for 5 min, then in a 50% dilution of Clorox liquid bleach (final concentration of 2.6% NaOCl) and sterile deionized water containing several drops of detergent (Ivory liquid dishwashing soap) for 20 min, followed by three rinses in sterile deionized water. Seeds were cultured in petri dishes containing 0.1-strength BDS medium (B5 as modified by Dunstan and Short, 1977) [letter B, number 5,] plus 3% (wt/vol) sucrose. Dishes were wrapped with Parafilm and incubated at 28.5° C with continuous light (cool white fluorescent bulbs) at 15 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Seeds germinated after 14–16 d. Cotyledons were excised from seedlings at the petioles, and each was divided transversely into two explants, producing four explants per seedling. Explants from one seedling were used as one experimental replicate.

Induction and enlargement of buds. Explants from 14–16-d-old seedlings were lightly pressed ventral side down onto bud induction medium (primary medium) in 20 × 100-mm petri dishes, 4 seedlings per dish (16 explants per dish). Dishes were wrapped with Parafilm and incubated at 28.5° C with continuous light (Phillips and Hubstenberger, 1985) at 15 $\mu mol \cdot m^{-2} s^{-1}$ for 14 d. Primary medium contained MS salts (Murashige and Skoog, 1962), L2 vitamins (Phillips and Collins, 1979), 3% (wt/vol) glucose (Phillips and Hubstenberger, 1985), 0.5 mg/L (2.9 *μM*) IAA, 2 mg/L (8.9 *μM*) BA, and 0.8% (wt/vol) Phytagar TC grade (Life Technologies, Gaithersburg, MD), pH 5.7. After 14 d incubation, the number of explants with buds was recorded. Explants were transferred to second-stage medium for bud enlargement during an additional 14 d of incubation. Second-stage medium in 25 × 100-mm petri dishes contained MS salts, L2 vitamins, 3% glucose, 0.8% Phytagar (TC grade), 2 mg/L (8.9 *μM*) BA, and 2 mg/L (6 *μM*) GA (potassium salt; Sigma Chemical Co., St. Louis, MO), pH 5.7. GA and $AgNO_3$ when included (5 mg/L; 29.4 *μM*), were prepared as sterile solutions and added to autoclaved media after cooling but before solidification with thorough mixing.

Shoot elongation. Rosettes and enlarged bud clusters that developed on second-stage media were excised from explants with cotyledon tissue attached and incubated on the third-stage medium for 1 month at 28.5° C with continuous light to enhance shoot elongation. Several third-stage media were tested. These media in Magenta GA7 boxes or 25 × 100-mm petri dishes contained MS salts, Nitsch's vitamins (Nitsch, 1969), 3% (wt/vol) sucrose (Arroyo and Revilla, 1991) or glucose, 0.8% Phytagar (TC grade), and 1-naphthaleneacetic acid (NAA: 0.0, 0.1, 0.5, or 1.0 mg/L; 0.0, 0.54, 2.7, or 5.4 *μM*), pH 5.7. In addition, a second incubation period on fresh dishes of second-stage medium was tested as a third-stage treatment.

Root production. Shoots were excised, usually with explant tissue attached at the stem base, and placed in Magenta boxes containing R1 medium consisting of MS salts, Nitsch's vitamins, 3% sucrose or glucose, 0.8% Phytagar (TC grade), and 1.0 mg/L (5.4 *μM*) NAA. After 1 month of incubation at 28.5° C with a 16-h photoperiod, shoots that did not develop roots were trimmed of any brown callus and incubated another month on R2 media, identical to R1 medium except for variation in NAA concentrations as described above. During incubation on R1 medium, additional shoots developed which were also transferred to R2 media.

Rooted shoot and plant maintenance. Each rooted shoot was transferred to a 3-oz plastic cup with soil (Terra Lite Metromix or Peat Lite), covered with a plastic bag, and placed either under cool white fluorescent lights in a 28.5° C incubator or under full spectrum fluorescent lights (Phillips C50) at room temperature with a 16-h photoperiod. Plantlets were watered with deionized water containing soluble plant fertilizer. When 4–6 cm tall, usually after 2–6 weeks, plants were acclimated gradually without a cover to ambient temperature and humidity, then transferred to 12-oz plastic cups and placed under full spectrum lights at room temperature. Some plantlets took 3 months to begin growing when first planted in soil. Within 1 month of growth in 12-oz cups, plants grew 15 cm tall or more and were large enough to transfer to pots in the greenhouse. They were already producing flowers or began to flower soon after placement in the greenhouse. During the next 3 months, fruit developed and matured.

Effect of agar source on bud induction. To determine if different commercial agars could improve bud induction, primary medium with 5 mg/L (29.4 *μM*) $AgNO_3$ was prepared either with 0.8% (wt/vol) Carolina Biological T.C. agar (Carolina Biological Supply Co., Burlington, NC), Phytagar Commercial Grade or Phytagar Tissue Culture Grade (Life Technologies). Each replicate was plated in a single dish (15 × 60 mm) with a minimum of 90 replications per treatment. Bud induction was evaluated after 14 d of incubation.

Bud induction by BA, thidiazuron, zeatin, and $AgNO_3$. Replicates of Joe E. Parker and NM 6-4 cotyledon explants were incubated individually in 15 × 60-mm petri dishes containing primary medium, with or without 5 mg/L (29.4 *μM*) $AgNO_3$. To determine the bud induction potential of different cytokinins, the medium also had either 2 mg/L (8.9 *μM*) BA, 5 mg/L (22.7 *μM*) thidiazuron (TDZ, Nor-Am Chemical Co., Wilmington, DE), or 5 mg/L (22.8 *μM*) zeatin (Sigma). Zeatin was filter-sterilized and added to autoclaved medium. Ten replicates per treatment, each replicate consisting of four cotyledon halves from one seedling, were incubated at 28.5° C with continuous light for 14 d. Explants with buds were transferred to second-stage medium containing 2 mg/L (6 *μM*) GA and the same cytokinin as in primary culture, each at 2.0 mg/L (8.9 *μM* BA, 9.1 *μM* TDZ, or 9.1 *μM* zeatin); half of the dishes had medium further supplemented with 5 mg/L (29.4 *μM*) $AgNO_3$. After incubation on second-stage media, explants were transferred to Magenta boxes containing one of three rooting media, two of which consisted of MS salts, L2 vitamins, 3% sucrose, 0.6% Phytagar, and either 0.5 mg/L (2.7 *μM*) NAA or 0.5 mg/L (2.9 *μM*) IAA. The third rooting medium contained half-strength MS salts, L2 vitamins, 1.5% sucrose or glucose, 0.6% Phytagar, and 0.1 mg/L (0.54 *μM*) NAA. Incubation was at 28.5° C with 16 h photoperiod for 1 month. All viable explants were transferred to R2 media with Nitsch's vitamins for another month of incubation. Rooted shoots that developed by these treatments were potted in soil, covered with a plastic bag, and placed at 28.5° C with 16 h photoperiod until they were large enough to transfer to the greenhouse.

Results and Discussion

Effect of Agar Quality on Bud Induction

In repeated experiments, cotyledon explants on primary medium, with or without $AgNO_3$ and solidified with Carolina Biological T.C. agar, had a tendency to swell during the first 14 d of incubation and developed only a few buds. Bud induction was as low as 0–10%, and buds were small. Few of the buds on such swollen explants enlarged on second-stage media, and eventually most explants were discarded.

Commercial agars are available in different grades of purity, and the purification processes of different manufacturers may leave residues noxious to pepper explants. To determine if agar quality was a factor in bud induction, primary medium with $AgNO_3$ was prepared with Carolina Biological agar, and bud induction on it was compared with that on the same medium made with Phytagar commercial grade. Only 33 of 100 replicates (33%) on medium solidified with Carolina Biological agar developed buds, and all of the explants were swollen and appeared to suffer from hyperhydricity (Table 1). In comparison, of 90 replicates on medium solidified with Phytagar commercial grade, 47 (52%) developed buds that were large and numerous, forming clusters, whereas many of the remaining replicates had small buds. None of these explants were swollen. After incubation on second-stage medium with $AgNO_3$, some of the explants were transferred to rooting medium on which several shoots elongated and rooted. Because bud induction and growth were superior on medium with commercial grade Phytagar, the tissue culture grade of Phytagar was tested and produced even higher bud induction percentages (85%, Table 1). Phytagar tissue culture grade was used preferentially in subsequent experiments with pepper.

Bud Induction by Different Cytokinins and $AgNO_3$, and Plant Regeneration

We tested three cytokinins and $AgNO_3$ for their ability to induce buds on cotyledon explants of two chile pepper cultivars by incu-

TABLE 1

SHOOT BUD INDUCTION ON COTYLEDON EXPLANTS OF CHILE PEPPER CV. JOE E. PARKER ON PRIMARY MEDIUM[a] WITH DIFFERENT AGAR SOURCES.

Agar source	No. of replicates[b]	No. of replicates with buds	% Bud induction ± SE	Response
Carolina Biological T.C. agar	100	33	33 ± 5	Buds were few and small. Explants were swollen.
Phytagar commercial grade	90	47	52 ± 5	Buds were numerous, large, and clustered. Explants were not swollen. Many remaining explants had swollen bud initials.
Phytagar tissue culture grade	96	82	85 ± 4	Buds were numerous, large, and clustered. Explants were not swollen.

[a] Primary medium contained MS salts, 3% glucose, L2 vitamins, 0.5 mg IAA/L, 2.0 mg BA/L, and 5 mg $AGNO_3$/L. Agar was added at 0.8% (wt/vol).
[b] One replicate = four cotyledon halves from one seedling. Replicates were cultured in individual dishes. Bud induction was determined 14 d after culture began.

bating them on primary medium with BA, TDZ, or zeatin, with or without $AgNO_3$. Bud induction varied from 40–100% among the 12 treatment groups (Table 2). Joe E. Parker responded better than did NM 6-4. Cytokinins BA, zeatin, and TDZ in media without $AgNO_3$ were nearly equal in ability to induce buds on Joe E. Parker explants (90–100%). With $AgNO_3$ present, zeatin was less effective (50%) than BA (90%), an TDZ was comparable (80%) to BA for bud induction.

Only those explants with buds were transferred to second-stage media for shoot elongation. Half of the media contained $AgNO_3$ and half did not. After 7 d at 28.5° C with continuous light, controlled incubation was interrupted in the laboratory. The effect of the interruption on the eventual development of plants in this experiment was uncertain, although subsequent experiments at constant 28.5° C have produced plants after culturing on similar media, indicating that there was little effect except perhaps delayed shoot elongation. In this particular experiment, after an additional 12 d of incubation at 22° C, many of the explants had rosettes composed of large, amorphic structures that looked like lettuce leaves. Those on TDZ also had long, thin projections, some of which developed into true leaves. No shoots developed from any explants during second-stage culture.

Rosettes in each treatment group were excised from cotyledon tissue and distributed among three rooting media. Several shoots and rooted explants developed after 1 month on medium containing 0.5 mg NAA/L. Then, explants transferred to R2 media with 0.5 mg NAA/L developed more shoots, and roots developed on 10–15% of the explants. Not all rooted shoots grew after being transplanted to soil. Those that did were transferred to the greenhouse where they rapidly developed flowers and fruit. Twenty-three plants of Joe E. Parker and 6 plants of NM 6-4 were obtained and are listed by treatment in Table 3. The only treatment that consistently failed to produce plants in both cultivars was that in which $AgNO_3$ was absent in both primary and second-stage media (− − Ag), regardless of cytokinin. Seeds from plants in each treatment group were tested for germination and were viable, and their cotyledon explants subsequently treated with BA and $AgNO_3$ also produced shoots.

During the second month of rooting medium treatment, the explants previously treated with BA and $AgNO_3$ developed shoots, whereas those treated with TDZ developed shoots during the third month of rooting treatment. Although more numerous, shoots induced by TDZ were usually very small, 2–3 mm tall, with a thin stem which did not elongate further or develop roots. Those that did root survived

TABLE 2

BUD INDUCTION ON COTYLEDON EXPLANTS OF CHILE PEPPER CVS. JOE E. PARKER AND NEW MEXICO 6-4 ON MEDIA WITH DIFFERENT CYTOKININS, AND THE EFFECT OF $AgNO_3$

Primary medium[a]	No. of replicates[b]	No. of replicates with buds		% Bud induction ± SE	
		Joe E. Parker	NM 6-4	Joe E. Parker	NM 6-4
BA + $AgNO_3$	10	9	5	90 ± 5	50 ± 8
BA − $AgNO_3$	10	9	4	90 ± 5	40 ± 8
Zeatin + $AgNO_3$	10	5	5	50 ± 8	50 ± 8
Zeatin − $AgNO_3$	10	9	8	90 ± 5	80 ± 6
Thidiazuron + $AgNO_3$	10	8	5	80 ± 6	50 ± 8
Thidiazuron − $AgNO_3$	10	10	9	100	90 ± 5

[a] MS salts, 3% glucose, L2 vitamins, 0.8% Phytagar T.C. grade, 0.5 mg IAA/L, ± 5 mg $AgNO_3$/L, and either 2 mg BA/L, 5 mg zeatin/L, or 5 mg thidiazuron/L.
[b] One replicate = four cotyledon halves from one seedling. Replicates were cultured on individual dishes. Bud induction was determined 14 d after culture initiation.

TABLE 3

REGENERATION OF MATURE PLANTS FROM COTYLEDON EXPLANTS OF CHILE PEPPER CVS. JOE E. PARKER AND NEW MEXICO 6-4 AND THE EFFECTS OF DIFFERENT CYTOKININS AND $AgNO_3$

Cytokinin ± $AgNO_3$ in primary medium[a]	Cytokinin and GA, ± $AgNO_3$ in second-stage medium[b]	No. mature plants[c]	
		Joe E. Parker	NM 6-4
BA + Ag	BA, GA + Ag	3	1
	BA, GA − Ag	1	0
BA − Ag	BA, GA + Ag	1	1
	BA, GA − Ag	0	0
Zeatin + Ag	Zeatin, GA + Ag	0	0
	Zeatin, GA − Ag	1	1
Zeatin − Ag	Zeatin, GA + Ag	0	1
	Zeatin, Ga − Ag	0	0
TDZ + Ag	TDZ, GA + Ag	6	2
	TDZ, GA − Ag	6	0
TDZ − Ag	TDZ, GA + Ag	5	0
	TDZ, GA − Ag	0	0
Total		23	6

[a] Primary media contained MS salts, 3% glucose, L2 vitamins, 0.8% Phytagar, 0.5 mg IAA/L ± 5 mg $AgNO_3$/L, and either 2 mg BA/L, 5 mg zeatin/L, or 5 mg TDZ/L. Cotyledon explants were incubated for 14 d and then transferred to second-stage media. Number of replications are the same as in Table 2.

[b] Second-stage media contained MS salts, 3% glucose, L2 vitamins, 0.8% Phytagar, 2 mg GA/L, ± 5 mg $AgNO_3$/L, and either 2 mg BA/L, 2 mg zeatin/L, or 2 mg TDZ/L. Number of replications are the same as the number of responding replicates in Table 2.

[c] Shoots elongated and rooted on media containing MS salts, 3% sucrose, Nitsch's vitamins, and 0.5 mg NAA/L. Plantlets were potted in soil and incubated under cool white or full spectrum fluorescent lights for 1–2 months. Plants developed flowers and fruit within 1 month of placement in greenhouse.

transplanting better; thus, the TDZ-treated plants were better represented in Table 3. Only a few shoots developed on explants treated with zeatin.

Our results with BA were consistent with those in previous reports in which BA with IAA were used successfully to regenerate pepper plants (Gunay and Rao, 1978; Fari and Czako, 1981; Phillips and Hubstenberger, 1985; Agrawal et al., 1989; Arroyo and Revilla, 1991; Valera-Montero and Ochoa-Alejo, 1992), and in which BA was more effective than zeatin (Gunay and Rao, 1978; Arroyo and Revilla, 1991). Because Joe E. Parker produced more plants than did NM 6-4, it was selected for further studies of bud and shoot induction by $AgNO_3$ and BA. N^6-benzyladenine was preferred over TDZ because of its earlier stimulation of shoot development in cultures of the chile peppers. Regeneration of pepper plants by organogenesis from explants treated with TDZ was reported only recently (Szasz et al., 1995). Some bell pepper genotypes but not others responded positively to TDZ treatment in that study.

Plant Regeneration from Joe E. Parker Explants Treated with BA and $AgNO_3$

We investigated the effects of $AgNO_3$ on bud induction in primary medium and subsequent shoot development in second-stage medium by establishing four treatment groups consisting of the two different media, with or without $AgNO_3$, on which cotyledon explants were incubated. Initial shoot elongation results prompted the addition of a third culturing stage.

Induction and enlargement of buds. Twenty to 28 replicates, consisting of four half-cotyledon explants per replicate, were initiated on each primary medium treatment. Buds developed on 87–93% of the explants on medium with $AgNO_3$ (Table 4) and on 80–93% of the explants incubated on medium without $AgNO_3$, indicating that the presence of $AgNO_3$ had little effect during this stage of culture. Buds formed on one or both cut edges of the explants and occasionally along the blade and were clustered too closely to be counted individually (Fig. 1 *A*). They appeared 7–9 d after plating on primary medium, with or without $AgNO_3$. Explants were incubated for 14 d before transfer to second-stage medium, although without transfer, buds continued to enlarge for up to 21 d, at which time some were overgrown with callus.

Because explants were cut into smaller pieces bearing bud clusters which developed at one or both cut ends and sometimes along the blade, the number of explant pieces on second-stage and subsequent media varied among experiments. Consequently, rather than the number of explant pieces, the number of replicates per treatment group is given for relative comparison of data (Table 4). Although the number of explant pieces varied, few were discarded. To avoid subjective elimination at each culturing stage of potentially regenerative tissue, any explant piece with buds that were not overgrown with callus was retained. Explants overgrown with callus never regenerated well-developed shoots.

Half of the replicates on primary medium with $AgNO_3$ were placed on second-stage medium with BA, GA, and $AgNO_3$; the other half were placed on second-stage medium without $AgNO_3$. Replicates on primary medium without $AgNO_3$ were similarly distributed to second-stage media with or without $AgNO_3$. When transferred to second-stage media, explants were cut into smaller pieces bearing bud clusters which continued to enlarge during incubation on second-stage media with or without $AgNO_3$. After 14 d of incubation, rosettes that formed on each medium were qualitatively different. Rosettes from buds induced on primary medium without $AgNO_3$ that enlarged on second-stage media without $AgNO_3$ (− − Ag) were small, pale, often brown where they contacted the medium, and fell apart easily when lifted from the medium. Rosettes incubated on primary and second-stage media with $AgNO_3$ (+ + Ag) developed into larger, amorphic leafy structures that were dark green. Rosettes from buds induced on primary media with $AgNO_3$ and second-stage media without $AgNO_3$ (+ − Ag) were similar in appearance to those treated both times with $AgNO_3$ (+ + Ag). The largest number of rosettes developed from buds produced on primary media without $AgNO_3$ and second-stage media with $AgNO_3$ (− + Ag). They were dark green and full but not as large as those treated + + Ag (Fig. 1 *B*).

Shoot elongation. Very few shoots elongated during incubation on second-stage media (Table 4). The few shoots that did appear were not specific to any of the Ag treatments. To encourage additional shoot elongation, rosettes and enlarged bud clusters were transformed from second-stage to new media. Initially, explants were transferred to a rooting medium (R1) for elongation on the basis of reports that shoots appeared while on rooting medium (Gunay and Rao, 1978; Fari and Czako, 1981; Agrawal et al., 1989) or even after explants developed roots (Valera-Montero and Ochoa-Alejo, 1992), were potted in soil (Arroyo and Revilla, 1991; Ebida and Hu, 1993),

TABLE 4

INFLUENCE OF TIME OF $AgNO_3$ APPLICATION ON SHOOT DEVELOPMENT FROM COTYLEDON EXPLANTS OF CHILE PEPPER CV. JOE E. PARKER

Experiment	No. of replicates on primary medium, ± $AgNO_3$[a]	No. of explants	No. of explants with shoot buds	% Bud induction ± SE	No. of replicates on second-stage medium, ± $AgNO_3$	No. of shoots produced on subsequent culture steps[b]: Second stage	Third stage	Rooting (R1)	Total no. of shoots (no. shoots/explant ± SE)
1 (no third-stage culture)	24 + Ag	96	88	93 ± 3	12 + Ag	1	Not used	4	5 (0.1 ± 0.1)
					12 − Ag	0	Not used	6	6 (0.1 ± 0.1)
	24 − Ag	96	88	92 ± 3	12 + Ag	2	Not used	56[c]	58 (1.2 ± 0.1)
					12 − Ag	0	Not used	0	0

Experiment	No. of replicates on primary medium, ± $AgNO_3$	No. of explants	No. of explants with shoot buds	% Bud induction ± SE	No. of replicates on second-stage medium, ± $AgNO_3$	Second stage	NAA, mg/L: 0.1	0.5	1.0	R1, from third stage: NAA, mg/L: 0.1	0.5	1.0	Total no. of shoots (no. shoots/explant ± SE)
2 (third-stage culture with varied NAA levels)	20 + Ag	80	70	88 ± 4	10 + Ag	0	2	0	1	2	0	1	6 (0.2 ± 0.1)
					10 − Ag	0	1	2	1	1	1	1	7 (0.2 ± 0.1)
	20 − Ag	80	74	93 ± 3	10 + Ag	0	11	10	10	3	0	1	35 (0.9 ± 0.1)
					10 − Ag	0	0	0	0	0	0	0	0

Experiment	No. of replicates on primary medium, ± $AgNO_3$	No. of explants	No. of explants with shoot buds	% Bud induction ± SE	No. of replicates on second-stage medium, ± $AgNO_3$	Second stage	BA and GA, ± Ag as in second stage	R1	Total no. of shoots (no. shoots/explant ± SE)
3 (third-stage culture repeated on fresh second-stage medium)	28 + Ag	112	90	80 ± 4	14 + Ag	0	3	2	5 (0.1 ± 0.1)
					14 − Ag	0	3	1	4 (0.1 ± 0.1)
	28 − Ag	112	97	87 ± 3	14 + Ag	0	20	5	25 (0.4 ± 0.1)
					14 − Ag	0	0	0	0
4 (third-stage culture repeated second-stage medium)	24 + Ag	96	77	80 ± 4	12 + Ag	0	7	1	8 (0.2 ± 0.1)
					12 − Ag	1	5	0	5 (0.1 ± 0.1)
	24 − Ag	96	88	92 ± 3	12 + Ag	0	23	4	27 (0.6 ± 0.1)
					12 − Ag	0	2	0	2 (0.1 ± 0.1)

[a] One replicate = four cotyledon halves from one seedling = four explants. Primary medium contained MS salts, 3% glucose, L2 vitamins, 0.8% Phytagar T.C. grade, 0.5 mg IAA/L, 2.0 mg BA/L, and ± 5 mg $AgNO_3$/L. Explants were incubated 14 d and then transferred to second-stage medium.

[b] Second-stage medium contained MS salts, 3% glucose, L2 vitamins, 0.8% Phytagar T.C. grade, 2.0 mg BA/L, 2.0 mg GA/L, and ± 5 mg $AgNO_3$/L. Explants were incubated 14 d and then transferred to third-stage or R1 media. Third-stage media consisted of fresh dishes of second-stage medium (BA, GA, ± Ag), or were similar to R1 medium with different NAA concentrations. R1 medium contained MS salts, 3% sucrose or glucose, Nitsch's vitamins, 0.8% Phytagar T.C. grade, and 1.0 mg NAA/L. Third-stage and R1 treatments each lasted 1 month.

[c] Three-fourths of the 56 shoots appearing in this group were very small, about 1–3 mm tall.

or both. In experiment 1 (Table 4), incubation on R1 medium for 1 month did result in shoot elongation. Shoots appeared in three of the four treatment groups. Explants in the fourth group, − − Ag, fell apart when lifted from second-stage medium and were not transferred to R1 medium. This is the same and the only group that did not produce plants of either Joe E. Parker or NM 6-4 when three cytokinins were tested in the previous experiment (Table 3).

Most of the shoots were found in one treatment group (Table 4), that is, 56 of the 66 shoots in experiment 1 elongated on explants previously incubated on primary medium without $AgNO_3$ and second-stage medium with $AgNO_3$ (− + Ag). Multiple shoots developed on at least five explant pieces in this treatment group. The 56 shoots were regenerated from 12 replicates. Three-fourths of them, however, were very small (2–3 mm stem height) or were callus-encrusted and did not develop further. The high auxin concentration in R1 (1.0 mg NAA/L) may have suppressed shoot elongation. Investigators who reported the appearance of shoots while explants were on rooting media or after roots developed had used lower auxin levels (0.1–0.5 mg NAA/L). The 1.0 mg NAA/L concentration in R1 was chosen here for its ability to induce roots on Joe E. Parker shoots (*see* Root Development in next section). Explants treated + − Ag or + + Ag produced 6 and 4 shoots, respectively, several of which rooted.

Because so many of the − + Ag shoots developed poorly on R1 medium (Table 4, experiment 1), a third culturing stage was added before R1 treatment to allow for shoot elongation in medium with less auxin. Media tested for the third stage had the same composition as R1 but with 0.1 or 0.5 mg NAA/L. For comparison, 1.0 mg NAA/L (R1) was included. In experiment 2 (Table 4), the same pattern of shoot development occurred as in experiment 1, namely, that the − + Ag explants developed the most shoots (31), compared with only 4 shoots from + − Ag explants and 3 shoots from + + Ag explants during third-stage culture. The number of shoots counted among the − + Ag groups on three different NAA concentrations were nearly

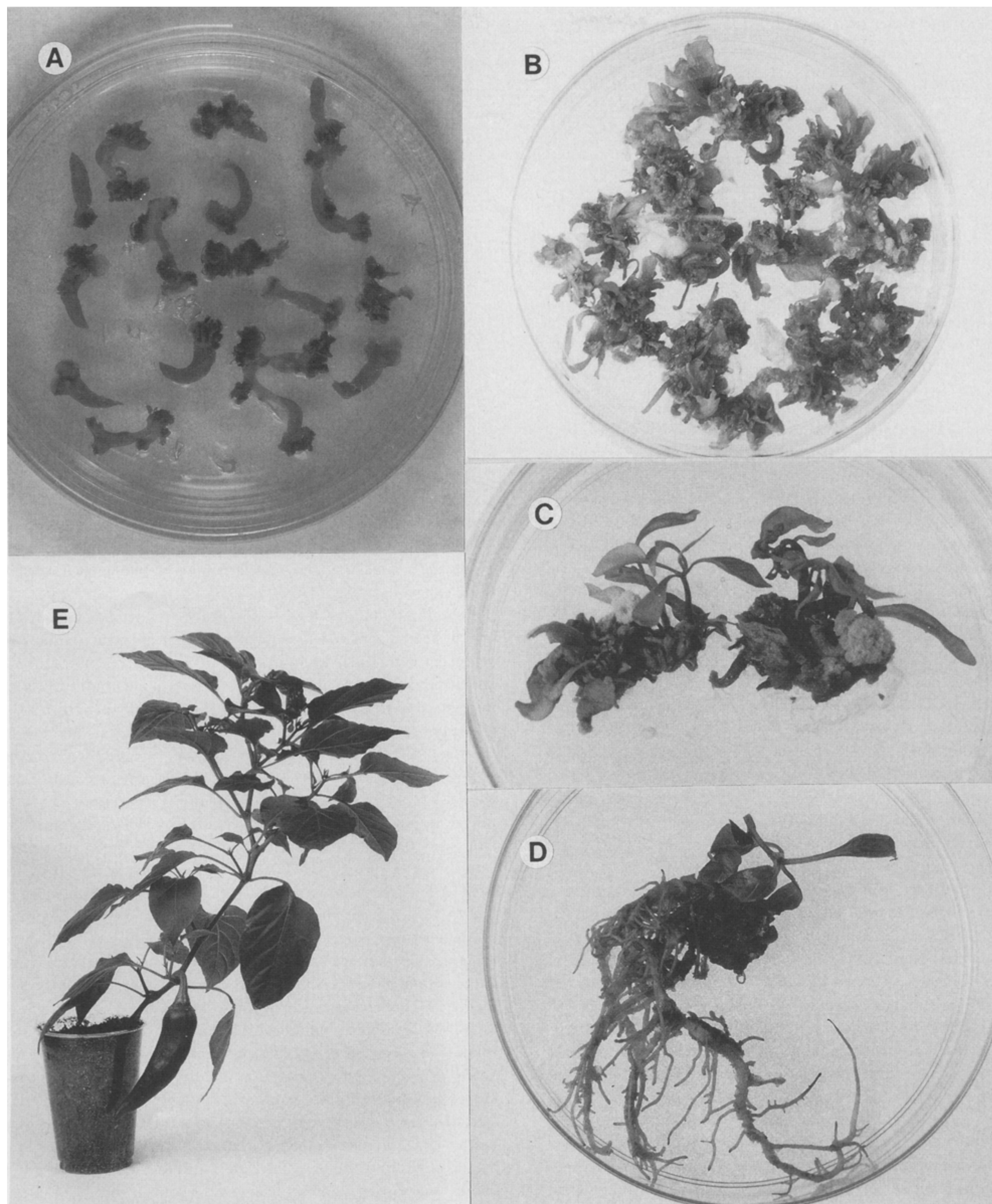

FIG. 1. Various stages of plant regeneration from cotyledon explants of chile pepper cv. Joe E. Parker. (*A*) Buds on explants after 13 d of culture on primary medium with BA and IAA. (*B*) Rosettes and bud clusters on explants after 14 d of culture on second-stage medium with $AgNO_3$. (*C*) Shoots produced on explants after 1 month on third-stage medium. (*D*) Plantlet (rooted shoot) after 1 month on R1 medium containing 1 mg NAA/L. (*E*) Mature regenerated plant with pod (treated first on primary medium without $AgNO_3$, then on second-stage medium with $AgNO_3$).

TABLE 5

SHOOT PRODUCTION FROM COTYLEDON EXPLANTS OF CHILE PEPPER CV. JOE E. PARKER WITH OPTIMIZED $AgNO_3$ TREATMENT

No. of replicates on primary medium − $AgNO_3$ and second-stage medium + $AgNO_3$[a]	No. of shoots on second-stage medium	No. of replicates per third-stage treatment	No. of shoots produced on third-stage media[b] (no. shoots/explant ± SE)				Total no. of shoots (no. shoots/explant ± SE)
				NAA, mg/L			
			BA + GA + Ag	0.0	0.1	1.0	
36 − + Ag	3	9	44 (1.2 ± 0.1)	25 (0.7 ± 0.1)	30 (0.8 ± 0.1)	28 (0.8 ± 0.1)	130 (0.9 ± 0.1)
40 − + Ag	5	10	20 (0.5 ± 0.1)	22 (0.6 ± 0.1)	17 (0.4 ± 0.1)	12 (0.3 ± 0.1)	76 (0.5 ± 0.1)

[a]MS salts, 3% glucose, L2 vitamins, 0.8% Phytagar, and for primary medium: 0.5 mg IAA/L + 2 mg BA/L; for second-stage medium: 2 mg BA/L + 2 mg GA/L $AgNO_3$/L. Explants were incubated 14 d on primary medium and 14 d on second-stage medium. One replicate = four cotyledon halves from one seedling. Data are from two experiments.

[b]Explants were incubated 1 month on third-stage media, either on new dishes with fresh second-stage medium (BA + GA + Ag) or on MS salts, 3% sucrose, and Nitsch's vitamins with NAA.

identical (11, 10 and 10), but shoots cultured on 0.1 mg NAA/L were better developed, indicating that the lower auxin concentrations favored shoot development.

In conjunction with this observation, another third-stage condition tested was an additional incubation period on fresh second-stage media. When explants were incubated 1 month on newly prepared dishes of the same second-stage media (a total of 6 weeks for second stage and third stage combined), shoots elongated (Table 4, experiments 3 and 4; Fig. 1 *C*). This result indicated that shoot development required more than a 2-week period on second-stage media. In experiment 3, 20 shoots developed from − + + Ag explants, compared with 3 shoots each from + − − Ag explants or from + + + Ag explants during third-stage culture. In experiment 4 there were 23, 5 (including a second-stage derived shoot), and 7 shoots, respectively, for the − + + Ag, + − − Ag, and + + + Ag treatments. Unique to this experiment was the appearance of two shoots from explants treated − − − Ag, which did not survive further culturing on R1 medium. Media were tested with either glucose or sucrose as the carbon source, with no significant difference in the shoot elongation results (data not shown).

Shoot elongation on different media in these four experiments showed the same pattern, that is, the largest number of shoots developed from − + Ag explants, whereas the smallest (almost negligible) number developed from − − Ag explants. The results of four treatment groups substantiated that not only was $AgNO_3$ required for shoot elongation, but that $AgNO_3$ was most effective in second-stage medium. Moreover, the presence of $AgNO_3$ in primary medium antagonized its potentiating effect in second-stage medium, so that its presence during both bud induction and bud enlargement resulted in a comparable number of shoots as developed after exposure to $AgNO_3$ in only primary medium.

The developmental influence of $AgNO_3$ may be qualitative in second-stage medium because its presence in primary medium did not significantly decrease bud induction. In the only published protocol for pepper regeneration that included $AgNO_3$, its presence in media was not shown to be required for regeneration (Valera-Montero and Ochoa-Alejo, 1992). In the original abstract, $AgNO_3$ was said to result in the best shoot and leaf differentiation (Jacobs and Stephens, 1990). Our results demonstrate that $AgNO_3$ is required at a specific developmental stage to promote shoot elongation and subsequent plant regeneration of at least one cultivar of pepper. The silver ion is a potent inhibitor of ethylene action (Beyer, 1976) and has been found to enhance shoot production in other plants (Purnhauser et al., 1987; Chi et al., 1990), but its mode of action during *in vitro* regeneration is still unknown. We tested silver thiosulfate and n-propyl gallate, which are also reported to be ethylene inhibitors, but obtained no beneficial effects during pepper regeneration from using these compounds (data not shown).

Four different third-stage media resulted in shoot elongation (Table 4). To compare which of these produced the most and best developed shoots, cotyledon explants were treated − + Ag in primary and second-stage media, respectively, then incubated 1 month on third-stage medium with 0.0, 0.1, or 1.0 mg NAA/L or on fresh dishes of second-stage medium with $AgNO_3$. Third-stage medium without NAA replaced that with 0.5 mg NAA/L (experiment 2) to compare whether R1-based medium without auxin was better than a longer incubation period on second-stage medium. The results of two experiments (Table 5) showed that the most numerous shoots developed after second-stage medium with $AgNO_3$ and on third-stage medium with BA + GA + Ag (44 shoots in first experiment) and/or lacking NAA (20 + 22 shoots in second experiment). These two treatment groups also had the largest shoots, most of which were 5–15 mm. The other two media, containing 0.1 or 1.0 mg NAA/L, produced generally smaller and less well-developed shoots. Variation in the data between these two experiments probably is real and may reflect the incomplete homozygosity of Joe E. Parker.

Third-stage shoots from experiments 2, 3, and 4 (Table 4) were excised with cotyledon tissue attached at the stem base and placed on R1 medium. During 1 month of incubation, additional but fewer shoots appeared with the same relative distribution among the four treatment groups as they did on third-stage medium. The total number of shoots produced on each medium in each treatment group is listed in the last column of Table 4, and these shoots were used in subsequent rooting studies.

Root development. The best concentration of NAA for root development on Joe E. Parker shoots was 1.0 mg/L in R1 medium (data not shown) which produced approximately 20% rooting. The tested concentrations were 0.1, 0.5, 1.0, and 2.0 mg/L. Rooting response with IAA was negligible. Use of NAA at 1.0 mg/L produced roots that were thick and branched (Fig. 1 *D*) whereas those produced with

TABLE 6

ROOTED SHOOTS PRODUCED FROM COTYLEDON EXPLANTS OF CHILE PEPPER CV. JOE E. PARKER

Experiment	No. of replicates ± $AgNO_3$ treatment on primary and second-stage media[a]	Total no. of shoots for rooting treatment	No. of rooted shoots produced on R1 and R2 media following third-stage culture[b]						Total no. of rooted shoots	% Rooted shoots ± SE (for ± Ag)
				R1		R2: NAA, mg/L				
						0	0.1	1.0		
1 (no third-stage culture)	12 + + Ag	5		1		0	0	0	1	
	12 + − Ag	6		2		0	0	0	2	
	12 − + Ag	58[c]		5		0	1	5	11	19 ± 5
	12 − − Ag	0								
			R1, from third-stage: NAA, mg/L			R1 repeated, from third-stage: NAA, mg/L				
			0.1	0.5	1.0	0.1	0.5	1.0		
2 (third-stage culture with varied NAA levels)	10 + + Ag	7	2	0	1	1	0	1	5	
	10 + − Ag	5	0	1	1	0	1	1	4	
	10 − + Ag	35	4	0	1	5	0	1	11	31 ± 8
	10 − − Ag	0								
				R1		R2: NAA, mg/L				
						0	0.1	1.0		
3 (third-stage culture repeated second-stage medium)	14 + + Ag	5		0		0	0	0	0	
	14 + − Ag	4		0		0	0	0	0	
	14 − + Ag	25		4		0	5	9	18	72 ± 9
	14 − − Ag	0								
4 (third-stage culture repeated second-stage medium)	12 + + Ag	8		4		0	0	0	4	
	12 + − Ag	6		5		0	0	0	5	
	12 − + Ag	27		6		1	2	10	19	70 ± 9
	12 − − Ag	2		0		0	0	0	0	

[a] Replicates are those described in Table 4. Media contained MS salts, 3% glucose, L2 vitamins, 0.8% Phytagar T.C. grade, ± 5 mg $AgNO_3$/L, and for primary medium: 0.5 mg IAA/L + 2.0 mg BA/L; for second-stage medium: 2.0 mg BA/L + 2.0 mg GA/L. Explants were incubated 14 d on each medium.

[b] Experiment 1 had no third stage culture; R1 medium followed second-stage medium. Third-stage culture in experiments 3 and 4 was on fresh second-stage medium. Explants in experiment 2 were incubated during third-stage culture on MS salts, 3% sucrose or glucose, Nitsch's vitamins, and 0.8% Phytagar T.C. grade with either 0.1, 0.5, or 1.0 mg NAA/L; R1 medium was repeated instead of following R1 with R2 medium. Each third-stage and rooting treatment was for 1 month. R1 and R2 media contained MS salts, 3% sucrose or glucose, Nitsch's vitamins, 0.8% Phytagar T.C. grade, and for R1: 1.0 mg NAA/L; for R2: 0, 0.1, or 1.0 mg NAA/L.

[c] Three-fourths of these shoots were small, about 2–3 mm tall.

IAA were thin and long, with little branching. There was no significant difference in rooting frequency when glucose and sucrose were compared as carbon sources (data not shown). Gunay and Rao (1978) described similar root development in pepper in response to NAA and IAA.

The treatment group that developed the most rooted shoots on R1 medium was the − + Ag group in all four experiments (Table 6). This is the same group that had the most shoots on third-stage media (Table 4). Although third-stage medium was not included in experiment 1, 5 of the 58 shoots produced in this group rooted, giving a rooting efficiency of 9% (Table 6). In the other three experiments, 5 of 35 shoots, 4 of 25 shoots, and 6 of 27 shoots rooted on R1 medium. The rooting efficiencies were 14, 16, and 22%, respectively.

Third-stage culture treatment may influence root development on R1 medium. Among the − + Ag explants in experiment 2, a nearly equal number of shoots developed on the three different NAA concentrations in third-stage media, but the most shoots to root were those that were on third-stage medium with 0.1 mg NAA/L (Table 6). In this group, 4 of 11 (36%) shoots rooted, whereas in the other two NAA groups, only 0–10% developed roots.

On some shoots, root initials may form in R1 medium with 1.0 mg NAA/L, but further development may be inhibited by the high auxin concentration. To test this possibility, unrooted shoots were transferred from R1 to R2 medium containing 0, 0.1 or 1.0 mg NAA/L. The + + Ag and + − Ag groups had few shoots to distribute among the three media, but some of them did root without apparent selectivity (Table 6, experiment 2). The group that performed the best was again the − + Ag group, the one which had the most shoots. More shoots tended to root on R2 medium with 1.0 mg NAA/L than with 0 or 0.1 mg/L (Table 6), indicating that some shoots may need a longer period of exposure to a higher concentration of auxin for root development. The additional rooting stage was beneficial to the − + Ag group because the production of rooted shoots on R2 medium more than doubled the rooting efficiency. When one compares the total number of rooted shoots with the total number of shoots tested (Table 6), the percentages increased from 9 to 19%,

14 to 31%, 16 to 72%, and 22 to 70% for experiments 1 through 4, respectively, with the additional rooting medium treatment.

Additional shoot elongation did not occur on R2 media. Well-rooted shoots were transferred to soil and grew into normal-appearing plants producing flowers and fruit (Fig. 1 *E*) with about 80% establishment success.

Overall Regeneration Efficiency

Using the optimal protocol described here for Joe E. Parker chile pepper, we obtained average bud induction frequencies of 88% across the data sets presented in Tables 1, 2, and 4. Shoot elongation occurred in about 50% of the explants responding with bud formation (Tables 4,5). With the optimized protocol, an average of 0.9 shoots elongated per initial explant used (Tables 4,5). About 71% of the shoots were rooted (Table 6). About 80% of the rooted shoots were established as viable plants in the greenhouse as noted earlier. Taken together, these results indicate that the overall regeneration efficiency was about 25% relative to the number of explants used. An average of 0.5 complete plants per cultured explant were successfully established in the greenhouse. Despite the improvements realized with silver nitrate in this protocol, the shoot elongation step continues to be the limiting step in pepper regeneration by organogenesis.

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