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# Morphogenesis and plant regeneration from tissue cultures of *Jatropha curcas*

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**Abstract**

Techniques for the regeneration of *Jatropha curcas* L. from various explants have been developed. Regeneration from hypocotyl, petiole and leaf explants was evaluated on a range of concentrations of zeatin, kinetin and $N^6$ benzyladenine (BA) either singly or in combination with indole-3-butyric acid (IBA). Higher regeneration from hypocotyl and petiole explants was obtained on BA with IBA than on zeatin- or kinetin- supplemented media. Leaf discs from the third expanding leaf exhibited higher regeneration potential than those from the fourth leaf. Independent of the explant type, direct adventitious shoot bud induction was recorded highest on MS medium with 2.22 μM BA and 4.9 μM IBA. Although the same BA concentration but with reduced IBA concentration (0.49 μM) proved effective in callus mediated regeneration from hypocotyl and leaf explants, the petioles required lower concentrations of the two growth regulators (0.44 μM BA and 0.49 μM IBA). Regenerated shoots could be rooted on growth regulator-free gelled full-strength MS medium. Following simple hardening procedures, the *in vitro*-raised plants could be transferred to soil and grown to maturity in the field.

*Abbreviations:* BA – $N^6$ benzyladenine; IAA – indole-3-acetic acid; IBA – indole-3-butyric acid; MS – Murashige & Skoog's (1962) medium; NAA – α-naphthaleneacetic acid

## Introduction

*Jatropha curcas* L. (Euphorbiaceae), a drought-tolerant perennial plant, has received extensive attention for the use of its seed oil as a commercial source of fuel (Takeda, 1982; Banerji *et al.*, 1985; Martin & Mayeux, 1985). Commonly known as sabudam/purging nut/physic nut, this plant is believed to have originated in South America (Brazil) and grows in all tropical regions. Kernels yield 46–58% of a semidry oil (iodine value 93–107) and contain mainly oleic (37–63%), linoleic (19–40%) and palmitic (12–17%) acids as constituent fatty acids (Anonymous, 1959). The oil is not edible due to the presence of a toxic substance, 'curcascine'; it is conventionally used in making soaps, candles, paints, lubricants and medicinally as a purgative.

The use of 'Curcas oil ' as a source of fuel has primarily been investigated in Thailand. To exploit this to the maximum extent, work is needed to select the best genotypes for oil content and quality. However, the seed oil productivity is very low since sabudam has not been improved as an oilseed crop and the basic knowledge for domestication has been generated recently. The genetic diversity in the natural population appears narrow since no remarkable morphological differences have been observed. Studies with mutation breeding and interspecific crossing have made little headway in increasing variability in this species (Sakaguchi *et al.*, 1987). A great number of the new approaches to genetic manipulation relied on the use of cell or tissue culture (Heinz & Mee, 1969; Larkin & Scowcroft, 1981) and this technique can be applied to crops like *J. curcas* where the genetic variability is limited.

Tissue culture studies in *Jatropha*, a large genus of herbs, shrubs and trees distributed in the tropics and sub-tropics, were confined to the species *Jatropha panduraefolia* and limited to endosperm culture (Srivastava, 1971; Johri & Srivastava, 1973; Srivastava & Johri, 1974). Recently, a protocol for high frequency regeneration from various explants of *Jatropha integerrima* has been developed (Sujatha & Dhingra, 1993). The present report describes a reproducible method for the differentiation of adventitious shoots directly and through callus derived from vegetative explants of *J. curcas*, which can be utilized for further improvement of the economic traits of this plant.

## Materials and methods

### *Explant sources*

Seedlings were grown in sterile vermiculite at 26–28 °C in light (30 μmol $m^{-2}$ $s^{-1}$) provided by cool, white fluorescent lights. Hypocotyl explants were collected from 2-week-old seedlings. Petioles were removed from leaves at the 4th and 5th nodes and leaves were collected from the 3rd and 4th nodes from the apex of branches of 2-year-old sexually mature plants grown in the field at the Directorate of Oilseeds Research, Hyderabad, India.

### *Surface disinfestation*

Hypocotyls, petioles and leaves were washed thoroughly with detergent (0.2% v/v Teepol) and running tap water. Surface disinfestation was effected with a 2.0% solution of sodium hypochlorite (1–1.2% available chlorine) for 15 min followed by four rinses in sterile glass-distilled water. Hypocotyls and petioles were cut transversely (0.5 cm), and placed with the proximal cut end in contact with the medium. Leaf discs (5 mm in diameter) were excised with a cork borer and placed with the abaxial face in contact with the medium.

### *Culture media and conditions*

Basal medium consisted of MS (Murashige & Skoog, 1962) salts and vitamins, 30 g $l^{-1}$ sucrose and 0.7% agar (Himedia, India). The medium was supplemented with a range of concentrations of different cytokinins : zeatin (0.046–22.8 μM), kinetin (0.046–23.2 μM), BA (0.044–22.2 μM) and auxins : NAA (0.54–53.7 μM), IAA (0.57–57.1 μM), IBA (0.49–49.0 μM) singly and in combination. Calluses were subcultured onto medium containing BA (0.44–4.44 μM) singly or with IBA (0.49–4.9 μM) for differentiation. The pH of each medium was adjusted to 5.8 ± 0.1 before pouring 15 ml into each 150 × 25 mm culture tube plugged with cotton wrapped in muslin cloth. The medium was autoclaved at 104 kPa at 120 °C for 20 min. All cultures were incubated at 26 ± 2 °C under a 16-h photoperiod using cool, white fluorescent lights (30 μmol $m^{-2}$ $s^{-1}$).

### *Acclimatization and field transfer*

Regenerated shoots (approximately 2–4 cm long) with well-developed roots were transferred to vermiculite in 6-cm diameter polybags kept in a tray and maintained at high humidity by covering with a glass plate for 15 days and then established in soil under field conditions.

### *Experimental design, data collection and analysis*

Experiments were set up in completely randomized design with 20 replicates per treatment and were conducted twice. Observations on the number of explants forming callus, shoots, roots and number of shoots per responding callus were recorded. Results were subjected to analysis of variance and significant differences in values were calculated where effects or interactions were statistically significant.

## Results and discussion

### *Induction of adventitious shoot buds*

Callus induction from hypocotyl, petiole and leaf explants was readily obtained within 2 weeks of incubation when auxins were used singly except on IAA-supplemented media which failed to elicit any response on hypocotyl and leaf explants. Roots were formed from callus at cut ends of hypocotyl explants in IBA (9.8 and 24.6 μM) or NAA (5.37 μM) supplemented media. Petiole explants produced white compact callus and leaf discs produced green compact callus in media supplemented with auxins alone but no organogenesis occurred. When cytokinins were used singly, only 9.12 μM zeatin induced shoots along with callus from hypocotyl explants while higher concentrations gave only callus. However, the response was very slow and overall frequency of regeneration was very low (data

not presented). Kinetin and BA incorporated alone in MS medium at various concentrations were unable to support growth of the explants and necrosis occurred. Further assessment of induction of morphogenesis was done on media supplemented with the three different cytokinins in combination with 4.9 μM IBA.

On media supplemented with a cytokinin and IBA (4.9 μM), callus induction was associated with direct shoot-bud formation. Hypocotyl and petiole explants swelled at the base after 7–10 days in culture and adventitious primordia appeared in the transition zone between the expanded and initial parts of the explant within 21–30 days. Shoot bud induction took place in three alternative pathways, irrespective of the explant source and growth regulator type/concentration. These were

- 5–6 small buds appearing individually (Fig. 1a),
- one elongated shoot, others as small buds (Fig. 1b) and
- elongated shoot and axillaries and others as protuberances (Fig. 1c).

Shoots from hypocotyls were surrounded by white callus and those from leaves and petioles by green callus. Buds originated directly from the explants while callus proliferated from the portion of the explant in contact with the medium. Histological studies in *Euphorbia pulcherrima* by Nataraja *et al.* (1973) revealed that some cortical cells of hypocotyls divided actively and developed into shoot buds.

MS medium with 2.22 μM BA and 4.9 μM IBA proved to be the most effective growth regulator combination for shoot bud induction in hypocotyl segments (Fig. 2). Zeatin (9.12 and 22.8 μM) plus IBA (4.9 μM) supplemented media also induced adventitious shoots. Some abnormal greenish structures resembling negatively geotropic roots were formed from hypocotyl segments on media supplemented with kinetin (2.32 and 4.65 μM) and IBA and also on low BA (0.44 μM) - IBA combinations. However, these structures failed to develop further. Roots were formed on media with a low concentration of zeatin (0.46 μM) and IBA through intervening callus. Similarly, BA-IBA combinations have been found to induce shoot formation from hypocotyl segments of *J. integerrima* (Sujatha & Dhingra, 1993). However, in the case of *Euphorbia peplus*, BA (2.5 or 10 μM) incorporated singly in half-strenth MS medium, was found suitable for adventitious shoot regeneration from internode segments (Tideman & Hawker, 1982). Caulogenic ability of petioles was exhibited at a wider range of growth regulator combinations than that of hypocotyl segments. BA (2.22 and 4.44 μM) and 4.9 μM IBA-supplemented media induced shoot buds from the base of petiole in all explants. However, increasing the BA concentration to 8.87 μM decreased the percentage of responding cultures (Fig. 2). Zeatin-IBA combinations failed to induce shoot formation, while kinetin (2.32–4.65 μM) with IBA proved amenable for organogenesis. De Langhe *et al.* (1974) have reported direct differentiation of buds on petiole explants of *Euphorbia pulcherrima*, another member of Euphorbiaceae. Callus started developing first on both cut ends with one end having bigger white callus while the other end had smaller reddish callus. Buds were mostly localized in the transition zone between the original explants and the bigger callus. However, in case of *J. curcas*, only the basal end touching the medium produced greenish callus while the distal end did not form callus.

In a subsequent experiment where different concentrations of BA (0.44, 2.22 and 4.44 μM) and IBA (2.46 and 4.9 μM) were compared to optimize the best growth regulator combinations for callusing and shoot regeneration from leaf explants, significant differences were observed in % callusing and shoot regeneration frequency between leaf discs from the two leaf types tested (Table 1). The leaf discs from the third expanding leaf gave a higher shoot regeneration frequency and at a wide range of concentrations tested. Maximum number (50%) of leaf discs producing adventitious shoots was recorded on medium supplemented with 2.22 μM BA and 4.9 μM IBA. However, in the case of discs from the fourth leaf, only 30% regenerated shoots at the highest concentration of BA used (4.44 μM BA and 4.9 μM IBA). This can be due to the physiological age of the explant in influencing organ formation as has been documented by George & Sherrington (1984).

*Morphogenesis in callus cultures*

*De novo* shoot bud formation through callus derived from hypocotyls, petioles and leaves occurred only when the callus was associated with a segment of the initial explant. Callus of hypocotyl origin produced shoots on various BA and IBA combinations tested but differed with respect to the percentage of calluses forming shoots and the mean number of shoots per callus (Table 2). Of the calluses derived from the three types of explants, leaf callus gave the highest response (100%) with 4.5 and 5.4 mean number of shoots per callus (4.44 μM BA and 2.46/4.9 μM IBA), while the highest number of shoots (10.7) was

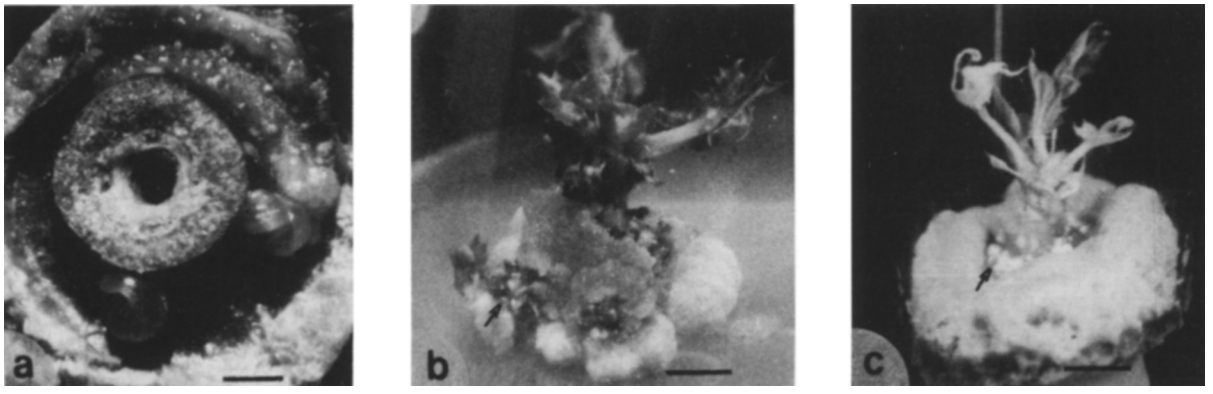


*Fig. 1.* Direct adventitious shoot bud induction from hypocotyl explants of *Jatropha curcas* on MS medium with 2.22 μM BA and 4.9 μM IBA after 4 weeks of culture. (*a*) Shoot bud(s) arising individually (Bar = 0.5 mm). (*b*) Elongated shoot and small buds (Bar = 5.0 mm). (*c*) Shoot with elongated axillaries and small buds as protuberances (Bar = 5.0 mm).

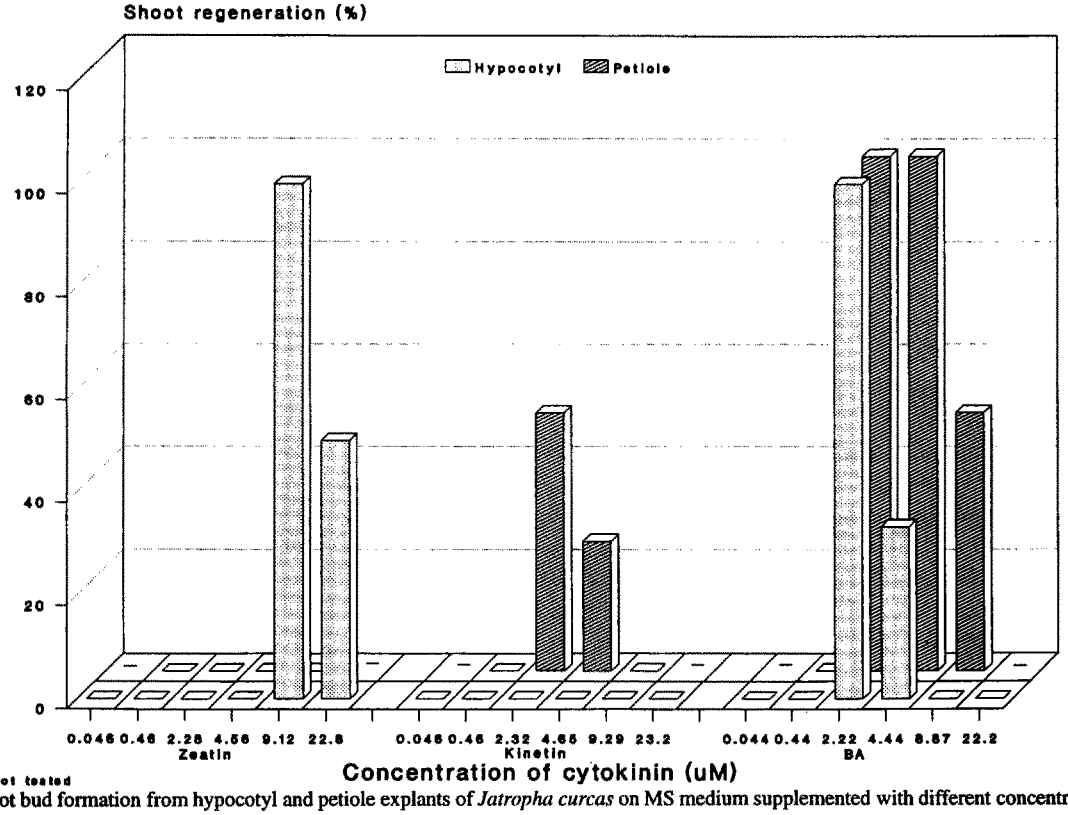


*Fig. 2.* Shoot bud formation from hypocotyl and petiole explants of *Jatropha curcas* on MS medium supplemented with different concentrations of cytokinins and IBA (4.9 μM) after 6 weeks of culture. LSD5% = 14%.

obtained on 2.22 μM BA and 2.46 μM IBA in 67% of the cultures (Fig. 3). The morphogenic competence of petiole-derived callus was limited to 0.44 μM BA alone or in combination with 0.49 μM IBA (Table 2). Variation among the explants in regeneration frequencies presumably may be due to predisposition of tissues from some organs to more rapid cell divisions than others and the fact that even closely associated tissues from one organ have different potentials. Irrespective of the source, the calluses produced shoots along with shoot clusters upon transfer to a medium with a higher cytokinin/auxin ratio than that used for callus induction. Further proliferation and elongation of the shoot buds could be achieved upon transfer to

*Table 1.* Influence of different concentrations of BA and IBA on callusing and shoot formation from leaf explants* of *Jatropha curcas.*

| Growth regulator concentration (μM) | | Third leaf | | Fourth leaf | |
|---|---|---|---|---|---|
| | | Explants forming | | Explants forming | |
| BA | IBA | Callus (%) | Shoots (%) | Callus (%) | Shoots (%) |
| 0.44 | 2.46 | 100 (90.0)[1] | 0 (4.1) | 45 (42.1) | 0 (4.1) |
| 0.44 | 4.90 | 90 (76.9) | 0 (4.1) | 50 (45.0) | 0 (4.1) |
| 2.22 | 2.46 | 100 (90.0) | 30 (32.6) | 100 (90.0) | 0 (4.1) |
| 2.22 | 4.90 | 100 (90.0) | 50 (45.0) | 50 (45.0) | 0 (4.1) |
| 4.44 | 0 | 8 (21.3) | 0 (4.1) | 50 (45.0) | 0 (4.1) |
| 4.44 | 2.46 | 100 (90.0) | 30 (32.6) | 50 (45.0) | 0 (4.1) |
| 4.44 | 4.90 | 100 (90.0) | 25 (30.0) | 92 (77.9) | 30 (32.6) |

Data scored after 6 weeks of culture
Experimental design - CRD with 3 factors. Treatments not effective are excluded.
LSD 5% = 14% and 13% for callusing and shoot regeneration, respectively.
* Significant at the 5% level according to the F-test with 1 and 47 DF.
[1] Figures in parenthesis are Arcsin angular transformed values.

*Table 2.* Effect of BA and IBA on shoot formation from callus of hypoctyl, petiole and leaf discs of *Jatropha curcas.*

| Treatments | | Hypocotyl | | Petiole | | Leaf | |
|---|---|---|---|---|---|---|---|
| BA (μM) | IBA (μM) | Shoot formation[1] (%) | No. of shoots[2] | Shoot formation (%) | No. of shoots | Shoot formation (%) | No. of shoots |
| 0.44 | 0 | 25 | 3.2bc | 11 | 1.4b | 0 | - |
| 0.44 | 0.49 | 50 | 2.1cd | 67 | 2.7a | 0 | - |
| 0.44 | 2.46 | 20 | 1.5d | 0 | - | - | - |
| 0.88 | 0.49 | 62 | 3.6b | 0 | - | - | - |
| 2.22 | 0 | 0 | - | 0 | - | - | - |
| 2.22 | 0.49 | 43 | 7.0a | 0 | - | 50.0 | 1.0c |
| 2.22 | 2.46 | 8 | 3.2bc | 0 | - | 67.0 | 10.7a |
| 2.22 | 4.90 | 3 | 1.2d | 0 | - | 43.4 | 1.7c |
| 4.44 | 2.46 | 21 | 1.7d | 0 | - | 100.0 | 4.5b |
| 4.44 | 4.90 | 7 | 2.0cd | 0 | - | 100.0 | 5.4b |

Completely Randomised Design (One-way ANOVA)
Means in a column followed by same letters are not significantly different according to Duncan's multiple range test at $\alpha = 0.05$
[1] % of calli regenerating shoots > 5mm
[2] No. shoots/regenerating calli
- Not tested

medium containing 2.22 μM BA. Although the concentration of BA found to be optimal for bud regeneration in *Jatropha curcas* was similar to that reported for *J. integerrima* (Sujatha & Dhingra, 1993); the presence of a segment of the initial explant was not necessary for callus-mediated shoot regeneration in the latter. Caulogenic callus has been reported in internode explants of *Euphorbia pulcherrima* (De Langhe *et al.*, 1974) and leaf explants of *E. hirta* (Baburaj *et al.*, 1987). Tideman & Hawker (1982) have reported callusing from leaf explants of three *Euphorbia* species; namely, *E. peplus, E. lathyris* and *E. tannensis.* Shoot regeneration did not occur on any of the BA-NAA combinations that were tried.

*Table 3.* Effect of auxins on rooting of *in vitro*- regenerated shoots of *Jatropha curcas*.

| Auxin concentration in MS basal medium (μM) | No. of days for rooting | Rooting* frequency (%) | Base callusing frequency (%) |
|---|---|---|---|
| No auxin | 8–10 | 88 | 0 |
| No auxin (Basal salts half strength) | 8 | 33 | 0 |
| NAA (2.69) | - | 0 | 33 |
| NAA (5.37) | - | 0 | 100 |
| NAA (10.74) | 10–14 | 62 | 50 |
| IAA (2.85) | - | 0 | 0 |
| IAA (5.71) | 7 | 17 | 0 |
| IAA (11.42) | - | 0 | 0 |
| IBA (2.46) | 7–8 | 17 | 0 |
| IBA (4.90) | 14 | 14 | 50 |
| IBA (9.80) | 9–11 | 33 | 100 |

Data scored after 4 weeks of culture.
* Number of shoots per treatment ranged from 15 to 40.
Coefficient of variation = 15.62%.

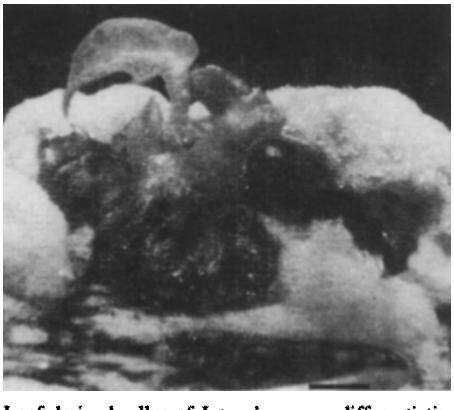

*Fig. 3.* Leaf-derived callus of *Jatropha curcas* differentiating shoots on MS medium with 4.44 μM BA and 4.9 μM IBA after two weeks of subculture (Bar = 0.5 mm).

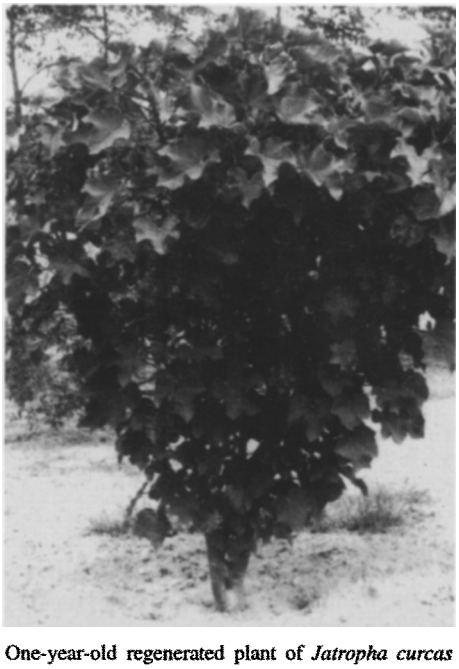

*Fig. 4.* One-year-old regenerated plant of *Jatropha curcas* established in the field.

### *Rooting and acclimatization of regenerated shoots*

Excised shoots of *J. curcas* (2–4 cm long) could be rooted on a wide range of auxins tested as well as on growth regulator-free MS medium (Table 3). Agar-gelled full-strength MS medium was found to be the best for rooting, while shoot bases turned black on liquid medium of the same composition. Though juvenile shoots rooted well, response was sporadic in the case of older shoots (data not presented). However, direct transfer to vermiculite and watering with a 4.9 μM IBA solution was successful in overcoming this problem partially. The regenerants established well in the

field with more than 80% success (Fig. 4). Plantlets obtained from this procedure and grown to maturity were normal and expressed no phenotypic variations. Plants grown in the field produced normal male and female flowers that developed into normal fruits. Seed yield, seed quality and number of seeds per plant were equivalent to plants propagated through seeds. Since no significant differences were observed for the various traits mentioned, quantitative data have not been presented.

The procedure described in this study represents the first successful attempt at regeneration of plantlets from the purging nut, *J. curcas,* and efforts are now underway for further enhancing the shoot bud regeneration frequency.

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