Appl Biochem Biotechnol (2012) 168:1729–1738
DOI 10.1007/s12010-012-9892-4

Elicitation of Andrographolide in the Suspension Cultures of *Andrographis paniculata*

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Received: 20 December 2011 / Accepted: 3 September 2012 /
Published online: 23 September 2012

Abstract *Andrographis paniculata* belonging to the family Acanthaceae produces a group of diterpene lactones, one of which is the pharmaceutically important—andrographolide. It is known to possess various important biological properties like anticancer, anti-HIV, anti-inflammatory, etc. This is the first report on the production of andrographolide in the cell suspension cultures of *Andrographis paniculata* by 'elicitation'. Elicitation was attempted to enhance the andrographolide content in the suspension cultures of *Andrographis paniculata* and also to ascertain its stimulation under stress conditions or in response to pathogen attack. The maximum andrographolide production was found to be 1.53 mg/g dry cell weight (DCW) at the end of stationary phase during the growth curve. The biotic elicitors (yeast, *Escherichia coli*, *Bacillus subtilis*, *Agrobacterium rhizogenes* 532 and *Agrobacterium tumefaciens* C 58) were more effective in eliciting the response when compared to the abiotic elicitors ($CdCl_2$, $AgNO_3$, $CuCl_2$ and $HgCl_2$). Yeast has shown to stimulate maximum accumulation of 13.5 mg/g DCW andrographolide, which was found to be 8.82-fold higher than the untreated cultures.

Keywords *Andrographis paniculata* · Andrographolide · Suspension cultures · Elicitation · Biotic · Abiotic

Introduction

Plants are considered to be the biofactories of planet Earth, from which about 100,000 low molecular weight compounds called secondary metabolites have been isolated. Plants

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produce secondary metabolites as part of the defense mechanism against microbes and higher organisms. These products are of immense use as potential drugs, nutraceuticals and food additives. Though many molecules have been synthetically designed, nature remains the source of highly sophisticated and biologically privileged compounds as they play a key role in increasing the survival fitness of living beings [1]. Numerous drugs and drug precursors in the current pharmacopoeia originate from plants. Limited yield of these bioactive compounds present a significant challenge for large-scale drug development. Plant cell and tissue culture systems can act as an alternate platform for large-scale production of bioactive compounds under controlled conditions [2].

Andrographis paniculata Nees belonging to Acanthaceae family, commonly known as 'Kalmegh', is widely distributed in India, Thailand, China and Malaysia. The main medicinal constituents are the andrographolide and related diterpene lactones like neoandrographolide and didehydroandrographolide. It has a broad range of pharmacological activities, viz. anti-inflammatory [3, 4], antiviral [5], hepatoprotective [6–9], cardiovascular [10], anticancer [11–16], anti-hepatitis [17, 18] and immunostimulant [19–21].

Cell cultures have a higher rate of metabolism because their initiation leads to faster proliferation of cells and a condensed biosynthetic cycle [22]. Further, plant cell cultures are not restricted by the environmental, ecological and climatic conditions, and cells can thus proliferate at higher growth rates than the whole plant in cultivation [23]. The secondary metabolite content in cell cultures can be further enhanced with elicitor treatment. Several studies have indicated that plant cultures are stimulated by elicitors ensuing rapid accumulation of secondary metabolites [24]. The phytochemical yield enhancement was observed in the various elicitor-treated medicinal plant cell cultures, e.g. *Abrus precatorius* Linn. [25], *Silybum marianum* (L.) Gaertn [26], *Commiphora wightii* [27], *Ammi majus* L. [28], *Ocimum basilicum* [29] and *Medicago truncatula* [30]. Hence, the present study was taken up with the objective to evaluate the effect of biotic and abiotic elicitors on the enhanced accumulation of andrographolide in the cell suspensions of *Andrographis paniculata*.

Materials and Methods

Materials

MS media and metal salts (Himedia), phytohormones (Duchefa Biochemie, Netherlands), andrographolide (Sigma), HPLC-grade chemicals (Merck) and *Bacillus subtilis* and *Escherichia coli* (Global Hospitals, Hyderabad) were used in this study. The *Agrobacterium rhizogenes* strain 532 (IMTECH, Chandigarh) and *Agrobacterium tumefaciens* strain C 58 were provided by Dr. Sumita Jha (Centre for Advanced Study, University of Kolkata) and baker's yeast acted as the source of yeast.

In Vitro Establishment of *Andrographis paniculata* Cultures

The seeds of *Andrographis paniculata* were obtained from CIMAP regional research centre, Hyderabad. The seeds were surface sterilised with 0.1 % (*w*/*v*) mercuric chloride for 3 min and later washed with sterile distilled water for five to six times and cultured on different basal media, viz. MS, B_5 and LS, supplemented with GA_3. All the cultures were maintained at 25±2 °C under 16/8-h (light/dark) photoperiod provided by the white fluorescent tubes (3,000 lux).

Initiation of Suspension Cultures

Explants from in vitro-germinated plantlets such as shoot and the root parts were evaluated for callus induction on MS media supplemented with various concentrations of phytohormones (6-benzylaminopurine (BAP) and 2,4-dichlorophenoxyacetic acid (2,4-D)). The suspensions of *Andrographis paniculata* were initiated by transferring the callus to MS liquid media with composition similar to that of callus proliferation and maintained for few days, to obtain homogenous cultures. The cultures were then used for initiation of suspensions.

Growth is determined by calculating the change in total cell suspension biomass along the plant's 'S' growth curve. Increase in biomass was calculated by harvesting the suspensions at regular intervals (3 days). The maximum biomass was estimated by weighing fresh biomass obtained after filtration. The andrographolide content was quantified by HPLC after each harvest.

Quantification of Andrographolide

Cells were harvested after filtration and oven dried at 60 °C. The dry cell biomass (0.5 g) was macerated in methanol into a fine paste with a mortar and pestle. The extract was filtered and the filtrate allowed to air dry for solvent removal. The obtained residue was then dissolved in HPLC-grade methanol (1 ml). This sample was filter sterilised before HPLC analysis and the andrographolide was calculated as milligrams per gram dry cell weight (mg/g DCW).

Andrographolide content in each sample was determined by HPLC equipped with a Shimadzu LC 10 AD pumps, SPD 10 A UV–Vis detector; the column used was Bondpak C 18 (3.9×300 mm) with a detection wave length of 223 nm. Mobile phase consisted of water, acetonitrile and methanol in the ratio of 55:30:15.

Elicitor Preparation

(a) Abiotic elicitors: Stock solutions of metal salts, i.e. $AgNo_3$, $CuCl_2$, $CdCl_2$ and $HgCl_2$ were prepared in distilled water at a concentration of 0.1 M. Varying concentrations of these salts, i.e.1, 2.5, 5, 7.5 and 10 mM, were added to the suspensions to study their effect on secondary metabolite production. All the salt solutions were filter sterilised before use.

(b) Biotic elicitors: *E. coli*, *B. subtilis*, yeast, *Agrobacterium rhizogenes* 532 and *Agrobacterium tumefaciens* C 58 microbial strains were used in the study. Forty-eight-hour-old cultures activated in nutrient broth were sonicated before addition to suspensions. The microbial cultures were filtered and the cell-free filtrate was used as the source of elicitor. Different concentrations in the range of 0.5–2.0 % (*v*/*v*) were added to the suspensions of *Andrographis paniculata*. The elicitors were added during the stationary phase of growth. The andrographolide content was monitored at a 24-h interval for 3 days.

Statistical Analysis

All the experiments were performed in triplicate, and the data were expressed as means± standard deviations. One-way ANOVA analysis followed by the Duncan's test was used to determine significant ($p \leq 0.05$) differences.

Results and Discussion

Secondary metabolites are used by the plants during adverse conditions like environmental alteration and pathogen attack. Moreover, their production also depends on the developmental stage of the plant as well as the geographical and climatic conditions. Since time immemorial, plants have been used as the best source for curing various diseases [31]. The usual practice is collection of plants from wild and harvesting them for extraction of bioactive compounds leading to their gradual extinction. In order to avoid extinction of local habitats, alternate methods, viz. plant tissue and cell culture techniques, are employed [23]. The suspension cultures are known to accumulate considerable amounts of secondary metabolites, which can be further enhanced by elicitation. Elicitor treatment was shown to affect the secondary metabolite biosynthesis in suspensions, root and whole plant cultures with commercial potential [32, 33]. Previous studies of elicitor treatment leading to increase in the secondary metabolite content in plants as well as the cell suspensions are *Plumbago rosea* [34], *Centella asiatica* [32], *Withania somnifera* [35], *Datura metel* [36] and *Gymnema sylvestre* [37].

The callus cultures were initiated from (3-week-old) plantlets, obtained from the in vitro-germinated seeds of *Andrographis paniculata*. Leaf, stem and root sections from the in vitro-grown plantlets were tried for callus induction (Fig. 1). The explants were inoculated initially on MS basal media supplemented with 2,4-D alone that did not allow callus proliferation. Hence, the media was enriched with another phytohormone, i.e. BAP, at different concentrations (Table 1; Fig. 2). Best response for callus initiation was observed on MS media supplemented with 2,4-D and BAP at a concentration of 2 and 0.4 mg/l, respectively. The efficacy of 2,4-D and BAP in callus induction has been proved in other medicinal plants, viz. *Ceropegia candelabrum* L [38] and *Adiantum capillus* [39].

Though callus induction was observed with all the plant parts, the response for each of them differed. The stem sections gave rise to compact callus whereas the root parts gave scant response. Leaf sections were preferred in terms of callus propagation rate and friability. Sharma et al. [40] in 1992 have reported that the leaves of *Andrographis paniculata* contain the highest amount of andrographolide (2.39 %), the most medicinally active phytochemical in the plant, while the seeds contain the lowest.

Andrographis paniculata suspensions were initiated in MS liquid media with concentration similar to the callus propagation media (2,4-D (2 mg/l) and BAP (0.4 mg/l)).

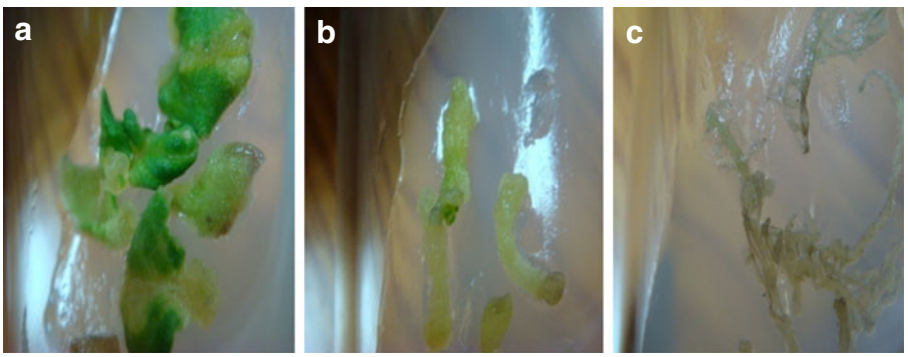

Fig. 1 Response of different explants for callus induction

Table 1 Phytohormone concentration effecting callus proliferation

Serial no.	2,4-D (mg/l)	BAP (mg/ l)	Response
1	0.5	–	–
2	1.0	–	–
3	1.5	–	–
4	2.0	–	–
5	3.0	–	–
5	0.5	0.1	–
6	0.5	0.2	–
7	1.0	0.2	–
8	1.0	0.4	+
9	1.5	0.2	+
10	1.5	0.4	++
11	2.0	0.2	++
12	2.0	0.4	+++
13	3.0	0.2	++
14	3.0	0.4	++

+ poor, ++ moderate, +++ good friable callus

Suspensions at 10 % inoculum concentration were employed to evaluate the growth pattern along with andrographolide production kinetics. The increase in biomass was analysed by harvesting the suspensions at a 3-day interval that continued until the culture reached decline phase, i.e. 24 days. The stationary phase for *Andrographis paniculata* suspensions is seen to span from the 12^{th}–18^{th} day. The maximum biomass was observed on the 15^{th} day (5.11 mg/g DCW). The present study reports andrographolide accumulation by the cell suspensions of *Andrographis paniculata* for the first time.

It was observed that the andrographolide production reached its peak (1.58 mg/g DCW) at the fag end of the stationary phase (18^{th} day). After the 18^{th} day, there was a significant decrease ($p<0.05$) in the andrographolide content (Fig. 3). From this observation, we can deduce that andrographolide production is not growth related but is necessarily produced under stress conditions or can be stimulated as a defense response. Whereas, the in vitro-grown roots of *Andrographis paniculata* have shown a maximum accumulation of

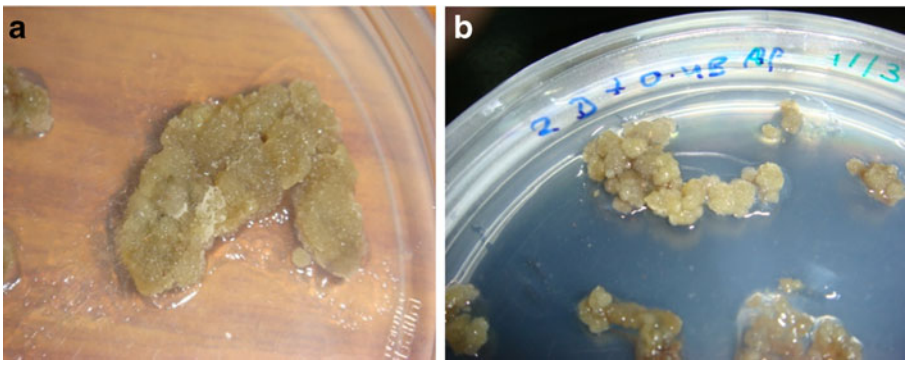

Fig. 2 **a**, **b** Friable callus on MS media supplemented with 2,4-D (2 mg/l) and BAP (0.4 mg/l)

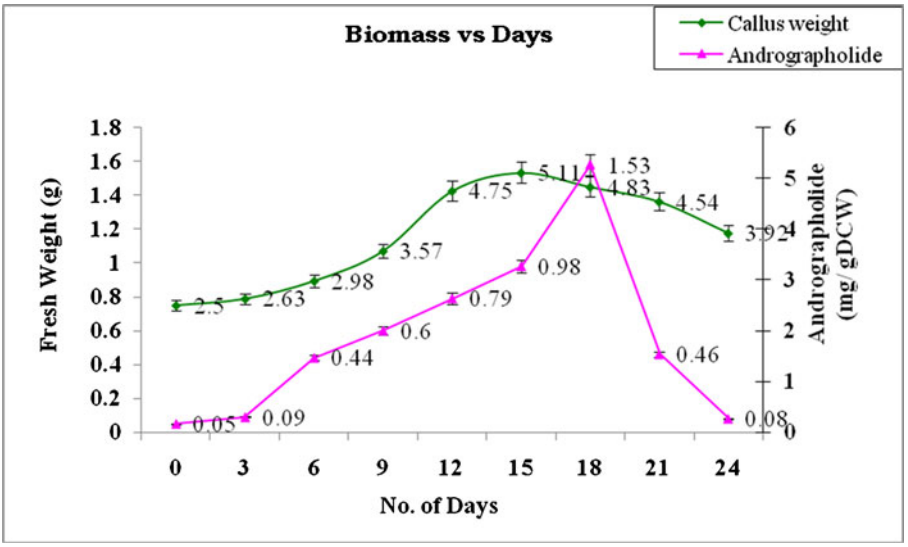

Fig. 3 Comparison between biomass increase and andrographolide production in the suspension cultures of *Andrographis paniculata*

andrographolide (72.86 mg/g DCW) after 28 days of culture in the report given by Praveen et al. [41]. From this, we can understand that by employing the suspension cultures of *Andrographis paniculata*, optimum production of andrographolide can be achieved in a short span.

As some of the secondary metabolites are defense related, different elicitors can be employed for their enhancement by stimulating the defense pathway. Environmental stresses, viz. abiotic (physical/chemical) and biotic (microbial), leading to enhanced secondary metabolite production are called elicitors [42]. Salts of heavy metals, viz. $CdCl_2$, $AgNO_3$, $CuCl_2$ and $HgCl_2$, were used as abiotic elicitors whereas, microbial homogenates, i.e. yeast, *E. coli*, *B. subtilis*, *Agrobacterium rhizogenes* 532 and *Agrobacterium tumefaciens*

Table 2 Study of optimum concentration and andrographolide production with different elicitors

Sample no.	Elicitor	Concentration	Andrographolide (mg/g DCW)	Fold increase
Abiotic elicitors				
1	$CdCl_2$	5 mM	6.34±0.85	4.14
2	$CuCl_2$	5 mM	2.42±0.08	1.58
3	$AgNo_3$	1 mM	2.35±0.11	1.53
4	$HgCl_2$	1 mM	1.86±0.10	1.21
Biotic elicitors				
1	Yeast	1.5 %	13.5±1.06	8.82
2	*E. coli*	1.5 %	8.3±1.10	5.42
3	*C-58*	1.5 %	3.16±0.34	2.06
4	*532*	1.5 %	4.2±0.70	2.74
5	*B. subtilis*	1.5 %	3.16±0.61	2.06
Control		–	1.53±0.08	–

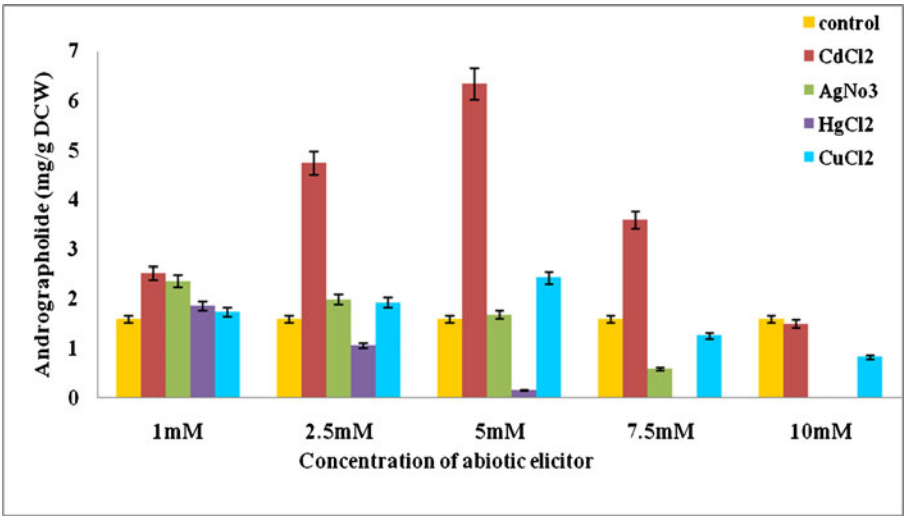

Fig. 4 Andrographolide accumulation against abiotic elicitor treatment

C 58, served as biotic elicitors. The optimum dose of elicitor was based on the maximum accumulation of andrographolide by the suspensions.

Elicitor concentration plays an important role in eliciting the response from cell cultures. The effect of abiotic elicitors was studied at different concentrations ranging from 1, 2.5… 10 mM. In case of $CdCl_2$ and $CuCl_2$, the optimum concentration was found to be 5 mM as against the lower optimum concentration of $AgNo_3$ and $HgCl_2$, i.e. 1 mM. The biotic elicitors on the other hand have shown maximum accumulation at 1.5 %, which was found to be optimal. High dosage of elicitor has been reported to induce hypersensitive response leading to cell death, whereas, an optimum level was required for metabolite production [33]. The effect of elicitor incubation time with the suspensions (24, 48 and 72 h) was analysed. The optimum incubation period was found to be 24 h for maximum accumulation of andrographolide (Table 2). Beyond the 24 h incubation period, for both biotic and abiotic elicitors, there was a decline in andrographolide accumulation in accordance to the report given by Fatima et al. [43] in *Phaseolus vulgaris*. Cultures treated during the early stationary phase have shown higher accumulation of andrographolide irrespective of the elicitor type similar to the study conducted by Zhang et al. in *Taxus yunnanensis* [44].

Among all the metal salts, $CdCl_2$ has shown a strong effect on andrographolide production at a concentration of 5 mM (Fig. 4). The andrographolide accumulation was found to be

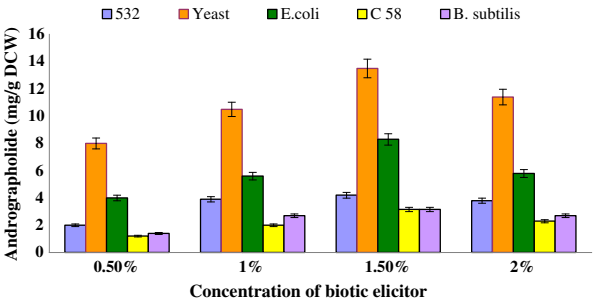

Fig. 5 Effect of biotic elicitors on andrographolide production from cell suspension cultures of *Andrographis paniculata*

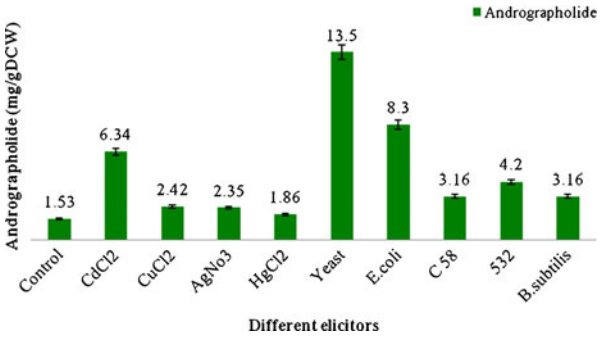

Fig. 6 Effect of biotic and abiotic elicitors on the accumulation of andrographolide from suspension cultures of *Andrographis paniculata*

6.3 mg/g DCW, whereas the control cultures accumulated 1.5 mg/g DCW. The accumulation order of andrographolide was found to be $CdCl_2$ > $CuCl_2$ > $AgNo_3$ > $HgCl_2$. There was a significant difference ($p<0.05$) in the amount of andrographolide accumulated by $CuCl_2$ and $AgNo_3$ in comparison to $HgCl_2$. The positive effect of $CdCl_2$ and $CuCl_2$ in enhancing the metabolites by activating the cell signalling pathways has been reported earlier [37, 45]. $AgNo_3$ and $HgCl_2$ have elicited positive response at lower concentration, as they are known to be highly toxic. Ag^+ is a known regulator of ethylene signal transduction pathway. Ethylene, a phytohormone, is known to regulate growth and development of the plant as well as the physiological reponses to wounds, insect attacks and even to small elicitor molecules [46]. $HgCl_2$ has been studied earlier for induction of PR proteins in various plants [47–50].

The microbial culture filtrates, viz. yeast, *E. coli*, *B. subtilis*, *Agrobacterium rhizogenes* 532 and *Agrobacterium tumefaciens* C 58 were used as the sources of biotic elicitors. The optimum dose was found to be 1.5 % (*v*/*v*) (Fig. 5). Later, as the concentration of the biotic elicitor increased, the andrographolide content decreased. In the present study, yeast has elicited maximum response from the suspension cultures at 13.5 mg/g DCW, which is 8.5-fold higher than the untreated sample. Yeast is a complex mixture which has amino acids, vitamins and minerals. The positive effect of yeast might be due to the presence of Zn, Ca and Co cations [51]. This was followed by *E. coli*, at 8.3 mg/g DCW, which was found to be 5.3-fold higher. *Agrobacterium rhizogenes* 532 accumulated 4.2 mg/g DCW, whereas there was no significant difference ($p>0.05$) in the amount of andrographolide production with *Agrobacterium tumefaciens* C 58 and *B. subtilis* (3.16 mg/g DCW). The effect of bacterial cell wall components and glycoproteins in eliciting positive response from plant cells has been reported [52–54]. In this study, yeast, *E. coli* and $CdCl_2$ were found to be very effective in enhancing andrographolide production (Fig. 6). These elicitors can pave way for large-scale production of andrographolide from cell cultures of *Andrographis paniculata* with commercial value.

Conclusion

Plants cell culture systems can be employed, when the secondary metabolite production is seen in undifferentiated system, contrary to the tissue specific accumulation. Plant cell cultures provide a qualitative, renewable source for production of large quantities of secondary metabolites at cheaper cost and less time, irrespective of the geographical conditions. The metabolite accumulation in cell cultures, in few cases, might exceed that of the

wild plants. Both biotic and abiotic elicitors were successful in eliciting response from the suspension cultures of *Andrographis paniculata*. The biotic elicitor, yeast, was able to induce eight fold increase in the accumulation of andrographolide in comparison to the untreated cultures. This study could pave the way for establishing a continuous and higher production of andrographolide, which has an enormous pharmacological importance.

Acknowledgments The financial assistance provided by the All India Council for Technical Education (AICTE), Government of India; is duly acknowledged.

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