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Biological changes in suspension cultures of *Taxus cuspidata* induced by dimethyl sulphoxide and ethanol

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Abstract

The biological changes in suspension cultures of *Taxus cuspidata* caused by dimethyl sulphoxide (DMSO) and ethanol, two commonly used solvents for water-insoluble elicitors, were investigated. The activities of peroxidase (POD) and superoxide dismutase (SOD) changed remarkably after the addition of small amount (0.4% (v/v)) of DMSO compared to those of the control culture at 4 h, however, they were less affected by small amount (0.4% (v/v)) of ethanol within 20 h. The biomass, cell viability, contents of intra/extracellular proteins did not change obviously when the amounts of DMSO and ethanol were below 1% (v/v) and 0.4% (v/v), respectively, but they varied significantly when the contents of DMSO and ethanol were 4% (v/v) and 1% (v/v), respectively. Obvious DNA fragmentation occurred in the case of ethanol at 2% (v/v), while no DNA fragments were observed in the case of DMSO at the same concentration level. It is inferred that DMSO below 1% (v/v) is a better solvent for investigating the effects of water-insoluble elicitors at a long-term contact, while ethanol less than 0.4% (v/v) is more suitable for a short-term contact.
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Keywords: DMSO; Ethanol; *Taxus cuspidata*; Viability; DNA fragmentation

1. Introduction

Dimethyl sulphoxide (DMSO) and ethanol are commonly used as solvents in biological and biochemical studies for dissolving water-insoluble drugs such as taxol and taxotere. However, the effects of DMSO and ethanol themselves on cells have not been elucidated yet. Recent studies showed that DMSO and ethanol have some biological effects on animal cells. Sharma et al. [1] reported that DMSO caused a reversible inhibition to telomerase, an enzyme related with cell proliferation, in lymphoma cell line. Kinashi et al. [2] suggested that DMSO have protective effects on animal cells against irradiation. Pagan et al. [3] showed that DMSO could abolish vimentin synthesis, inhibiting cell growth. Oberdoerster and Rabin [4] claimed that ethanol could increase caspase activity leading to apoptosis in rat cerebellar granule cells. However, the understanding of the effects of DMSO and ethanol on plant cells is still very limited compared to that on animal cells.

Taxol is a very effective antineoplastic chemotherapeutic agent against a wide variety of tumors especially ovarian and breast [5]. The recognized function of taxol is preventing microtuble from disintegration [6]. In addition, inducing apoptosis of tumor cells might be another mechanism of taxol in cancer therapy [7–9]. Apoptosis is a gene-directed active form

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of cell death in animals and plants in development or as a result of cellular responses to environmental stresses [10]. Our previous experiments showed that the introduction of additional taxol in suspension cultures of *Taxus cuspidata* induced an apoptosis [30]. However, it was observed that the ratio of apoptotic cells to total cells did not increase with the increase in concentration of the added taxol as expected from the results in animal cells. It is thus inferred that taxol might take a function of activating the genes relevant to apoptosis in plant cells, rather than acting on microtuble as observed in animal cells.

In animal cells taxol could induce direct bcl-2 phosphorylation [7–9] and p53 modulation [10,11], leading to an apoptosis. There is the increasing evidence that reactive oxidative intermediates (ROI) mediate bcl-2 phosphorylation and modulation p53 [12–16], so it is presumed that the added taxol into the suspension cultures might influence ROI levels. In investigation of cell apoptosis in suspension cultures of *T. cuspidata* induced by the added taxol, we found that the solvents used for dissolving taxol strongly affected the physiological states of cells. Therefore, the effects of solvents should be considered for a better understanding of the mechanism of cell apoptosis induced by added taxol.

In this work, our attention was paid to the biological effects of DMSO and ethanol on the suspension cultures of *T. cuspidata* so as to provide some useful information for selecting suitable solvents in study of cell apoptosis induced by water-insoluble elicitors in plants.

2. Materials and methods

2.1. Chemicals

RNAase A, EDTA, β-mercaptoethanol, dithiothreitol (DTT), cetyltrimethyl ammonium bromide (CTAB), 2,3,5-triphenyltetrazolium chloride (TTC), agarose and Coomassie brilliant blue G-250 were purchased from Sigma. All other chemicals used were of analytical grade and obtained commercially.

2.2. Culture conditions

The cell line from young stems of *T. cuspidata* was sub-cultured on modified B_5 solid medium containing sucrose (25 g/l), agar (0.9% (w/v)), naphthylacetic acid (2 mg/l) and 6-benzyl aminopurine (0.15 mg/l) at 25 °C in dark [17]. Cell suspensions were sub-cultured every 10–12 days for totally five generations in a modified B_5 liquid medium of the same compositions with the solid medium except the absence of agar. Cultures (120 ml) at pH 5.8 were maintained at 25 °C in dark with continuous shaking at 100 rpm in 500 ml flasks. Fresh cells (3.0 g) from suspension cultures of the fifth generation were inoculated into 50 ml fresh modified B_5 medium in a 250 ml Erlemeyer flash. Cell samples were collected at the late exponential phase of cell growth (day 15) for various analyses. All the experimental data were the average of triplicate samples and the errors were within ±10%.

2.3. Extraction and assay of enzymes

2.3.1. Intra/extracellular enzyme extraction

Fresh cells (0.5 g) were grinded in liquid nitrogen with mortar and pestle, then 3 ml 0.1 M phosphate buffer of pH 7.2 containing EDTA (2 mM), DTT (4 mM) and polyvinylpyrrolidone (0.4% (w/v)) was added. The mixture was homogenized at 4 °C and centrifuged at 10,000 × *g* for 10 min. The supernatant was collected for analyses of the intracellular enzymes.

For extraction of the extracellular enzymes, suspension cultures (3 ml) were centrifuged at 4 °C and 10,000 × *g* for 10 min. The supernatant was collected as the extract of extracellular enzymes.

2.3.2. Assays of enzymes and total soluble proteins

Peroxidase (POD), superoxide dismutase (SOD) and total soluble proteins were assayed following the method of Zhang et al. [18]. One unit of POD activity was defined as the amount that caused an absorbance change of 10 (intracellular) or 1 (extracellular) per minute at 470 nm. One unit of SOD activity was expressed as the amount that inhibited 50% of the colorimetric reaction.

2.4. Measurement of biomass

Suspension cultures (50 ml) were filtered through a funnel at reduced pressure. The cells were collected, lyophilized and weighed.

2.5. Cell viability

Cell viability was assayed using 2,3,5-triphenyltetrazolium chloride (TTC) following the method of Iborra et al. [19] and expressed as the absorbance of the sample treated with DMSO or ethanol at 485 nm/g of dry biomass.

2.6. Extraction and analysis of DNA fragments

Total DNA was extracted following the method of Dellaporta et al. [20] with a slight modification. Fresh cells (0.2 g) were grinded in liquid nitrogen with mortar and pestle, then the cell powders were transferred to a sterilized Eppendorf tube containing 600 μl buffer consisting of CTAB (2% (w/v)), Tris–HCl (10 mM) of pH 8.0, EDTA (20 mM) of pH 8.0, NaCl (1.4 mM) and β-mercaptoethanol (2% (v/v)) at 65 °C. The mixture was shaken slightly to get well mixing and then incubated at 65 °C for 30 min and centrifuged at 10,000 × g for 20 min. The aqueous phase (the upper layer) was extracted with equal volume of chloroform:isoamylol (24:1 (v/v)), then the supernatant was collected and mixed with equal volume of pellet buffer of pH 8.0 consisting of CTAB 1% (v/v), Tris–HCl (50 mM) and EDTA (10 mM) at 65 °C for 30 min to precipitate DNA. The total DNA was collected by centrifugation at 3000 × g for 10 min. The pellets were collected and re-suspended in 0.5 ml high-salt TE buffer (pH 8.0) containing Tris–HCl (10 mM), EDTA (1 mM) and NaCl (1.0 M). DNA was precipitated by addition of twice volume of ethanol at −20 °C for 1 h, then centrifuged and washed with 70% ethanol and dried at 37 °C. Finally, the total DNA was dissolved in 30 μl TE buffer of pH 8.0 consisting of Tris–HCl (1 mM) and EDTA (10 mM). RNase A (100 μg/ml) was added to digest RNA at 37 °C for 30 min. The DNA samples were run on 1.2% (w/v) agarose gel stained with ethidium bromide (0.5 μg/ml) to observe the DNA fragments under UV illumination.

3. Results

3.1. Effects of DMSO and ethanol on enzyme activity

POD and SOD are protective enzymes of plant cells against a variety of physical, chemical and biological stresses by regulating the concentrations of O_2^- and H_2O_2 [21,16]. Table 1 lists the variations of POD and SOD activities after 4 and 20 h in the cases of DMSO and ethanol, respectively. SOD activity increased after addition of DMSO compared to that of the control culture after 20 h except in the case of 2% (v/v). Extra-

Table 1
Effects of DMSO and ethanol on activities of superoxide dismutase (SOD) and peroxide (POD)

Sample	Content (% (v/v))	Time (h)[a]	Activity		
			Extracellular POD[b]	Intracellular POD[c]	Intracellular SOD[d]
Control	0	4	4.7	49.0	6.2
		20	4.8	49.1	6.5
DMSO-treated	0.4	4	6.5	51.2	7.2
		20	4.5	51.9	7.1
	2	4	6.7	70.7	6.6
		20	5.0	27.3	4.2
Ethanol-treated	0.4	4	4.9	50.7	6.0
		20	5.0	51.8	6.3
	2	4	6.4	36.9	3.6
		20	4.9	32.5	5.5

[a] Solvents were added into the suspension cultures of *T. cuspidata* at day 15.
[b] Extracellular POD activity was expressed as U/ml extracellular medium. One enzyme unit (U) was defined as the amount that caused an absorbance change of 1 min^{-1} at 470 nm.
[c] Intracellular POD was expressed as U/g cells (dry wt.). One enzyme unit (U) was defined as the amount that caused an absorbance change of 10 min^{-1} at 470 nm.
[d] Intracellular SOD activity was expressed as U/mg cells (dry wt.). One enzyme unit (U) was defined as 50% inhibition of the colorimetric reaction.

cellular POD activity obviously increased at 4 h then decreased approximately to the control level. However, intracellular POD activity first increased then decreased remarkably. Ethanol had almost no effect on activities of POD and SOD at low content (0.4% (v/v)). However, at high content (2% (v/v)) ethanol obviously inhibited SOD and intracellular POD. The change of extracellular POD activity was similar to that in the case of DMSO at the same concentration level. It is interesting to note that the activities of POD and SOD were less affected within 20 h at an ethanol content of 0.4% (v/v). Thus, ethanol is a better solvent for dissolving taxol and other water-insoluble elicitors in study of their effects on the relevant enzymes in plant suspension cultures.

3.2. Effects of DMSO and ethanol on biomass

Fig. 1 is the time course of biomass in the cases of ethanol and DMSO. At lower contents (below 0.4% (v/v)) of organic solvents, the biomass was almost unaffected compared to that of the control culture. However, at higher contents (over 4% (v/v)) of solvents, the biomass sharply decreased within the first 4 days. When the solvent content was moderate (1% (v/v)), the biomass was almost unaffected in the case of DMSO (Fig. 1a) but obvious decreased in the case of ethanol (Fig. 1b). This result indicates that ethanol was more toxic to cells compared to DMSO.

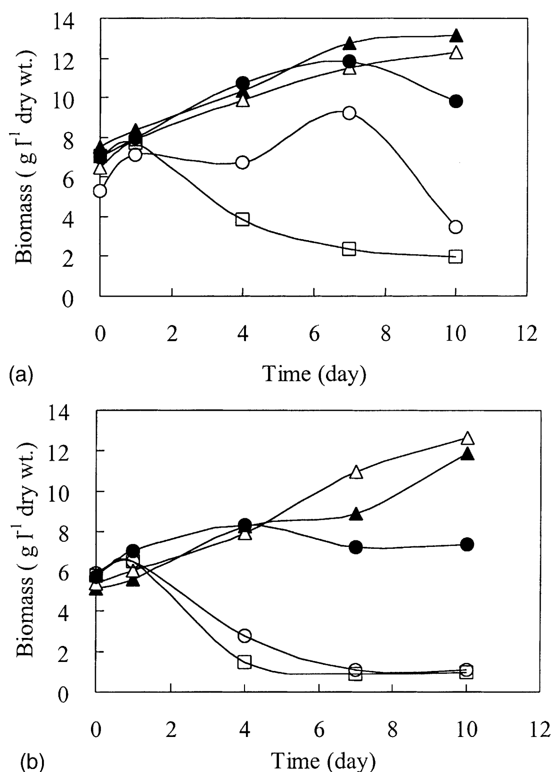

Fig. 1. Time course of biomass in the cases of DMSO (a) and ethanol (b). The solvents were added into the suspension cultures of *T. cuspidata* at day 15 after cultivation. (▲): Control culture. Solvent content (v/v): (△) 0.4%, (●) 1%, (○) 2%, (□) 4%.

3.3. Effects of DMSO and ethanol on cell viability

Fig. 2 is the time course of cell viability in the cases of ethanol and DMSO. In the case of DMSO (Fig. 2a), the cell viability was almost unaffected compared to that of the control culture when the DMSO content was below 2% (v/v). However, when the DMSO content was raised to 4% (v/v), the cell viability decreased severely.

In the case of ethanol (Fig. 2b), the cell viability slightly raised compared to that of the control culture when the ethanol content was below 0.4% (v/v). However, when the ethanol content was 1% (v/v), the cell viability decreased obviously. Further increase of ethanol content (over 2% (v/v)) reduced the cell viability to a very low level (below 4 absorbance/g dry wt.) at 4 days, indicating the death of almost all cells. This result further confirms the presumption that ethanol was more toxic to the cells.

3.4. Effects of DMSO and ethanol on intra/extracellular proteins

Fig. 3 is the time course of intra/extracellular proteins in the case of DMSO. When the DMSO content was below 2% (v/v), the contents of both intra- and extracellular proteins were less affected compared to those of the control culture. However, at a DMSO content of 4% (v/v) the content of extracellular proteins significantly increased (Fig. 3a) while the content of intracellular proteins decreased sharply especially at longer contact time (Fig. 3b).

Fig. 4 is the time course of intra/extracellular proteins in the case of ethanol. When the ethanol content was below 0.4% (v/v), the contents of both intra-

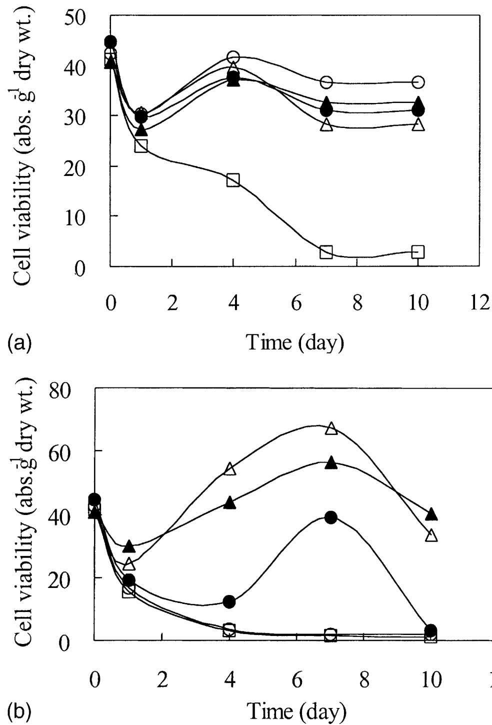

Fig. 2. Time course of cell viability in the presence of DMSO (a) and ethanol (b). Cell viability was expressed as the absorbance at 485 nm/g of biomass (absorbance/g dry wt.). (▲): Control culture. Solvent content (v/v): (△) 0.4%, (●) 1%, (○) 2%, (□) 4%.

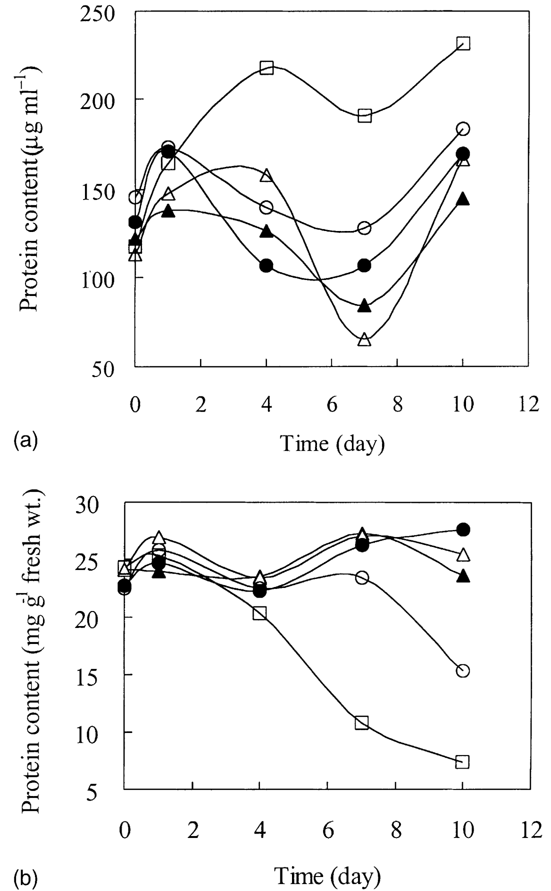

Fig. 3. Time course of extracellular (a) and intracellular (b) soluble protein contents in the presence of DMSO. (▲): Control culture. DMSO content (v/v): (△) 0.4%, (●) 1%, (○) 2%, (□) 4%.

and extracellular proteins were less changed compared to those of the control culture. However, when the ethanol content was over 2% (v/v), the contents of both intra- and extracellular proteins appreciably decreased. It is worth mentioning that at moderate ethanol content (1% (v/v)) the content of extracellular proteins increased compared to that of the control culture (Fig. 4a) whilst the content of intracellular proteins sharply decreased with time.

The more significant changes in contents of intra- and extracellular proteins in the case of ethanol is also in agreement with the more toxic feature of ethanol to cells than DMSO.

3.5. DNA degradation caused by DMSO and ethanol

Fig. 5 is the electrophoresis of the nuclear DNA (nDNA) in the cases of DMSO (lines 2 and 3) and ethanol (lines 4 and 5) at day 4 after addition of the solvents. When the ethanol content was 2% (v/v; line 5), longer DNA smear was observed, indicating the occurrence of a random DNA degradation. In contrast, at a DMSO content of 2% (v/v; line 3), no DNA smear was visible. Therefore, ethanol caused a severer damage to DNA cells than DMSO at higher concentration levels.

4. Discussion

The addition of DMSO or ethanol might lead to the generation of ROI as a cellular response to the adverse stimuli [22,23]. As ROI are toxic to cells, plant cells tend to eliminate them by the antioxidant enzymes

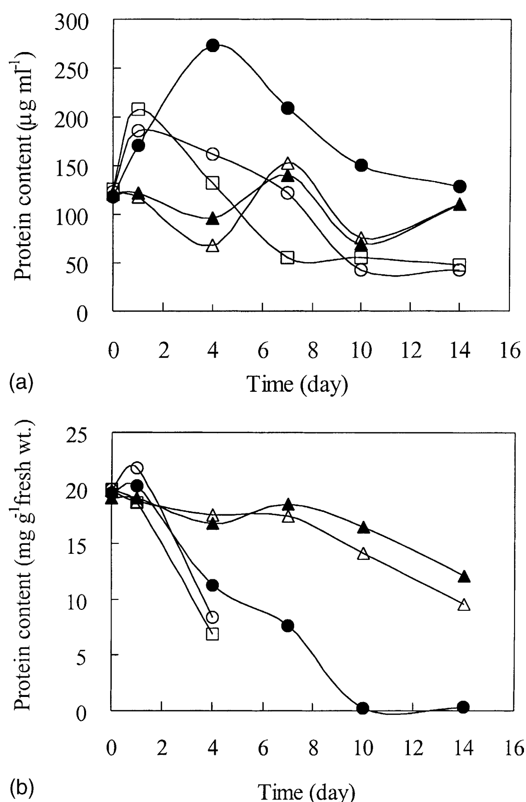

Fig. 4. Time course of extracellular (a) and intracellular (b) soluble protein contents in the presence of ethanol. (▲): Control culture. Ethanol content (v/v): (△) 0.4%, (●) 1%, (○) 2%, (□) 4%.

Fig. 5. Agarose gel electrophoresis of total DNA from the cultures added with DMSO or ethanol. Lane 1: intact DNA of control cells; lanes 2 and 3: intact DNA induced by DMSO at 0.4% (v/v) and 2% (v/v) after 4 days; lane 4: intact DNA induced by ethanol (0.4% (v/v)); lane 5: DNA smear induced by ethanol (2% (v/v)) after 4 days. DNA was isolated after harvest, separated on an agarose gel (1.5% (w/v)) by electrophoresis, stained with ethidium bromide and photographed under UV illumination.

such as POD and SOD. POD decomposes excess hydroperoxides such as hydrogen peroxide and participates in the cross-linking of lignin precursors to reinforce cells walls and SOD scavenges excess superoxide anions into hydrogen peroxides [22]. Therefore, the variations of SOD and POD activities reflect the ability of plant cells to withstand the unfavorable stimuli by the solvents added.

The increases in SOD and extra-POD activities at low content of DMSO at 4 h (Table 1) indicates that the *T. cuspidata* cells eliminated the excess superoxide anions and hydrogen peroxide caused by DMSO. However, SOD and extra-POD activities returned to their normal levels at ca. 20 h, showing that the cells began to adapt the existence of DMSO at low concentration level. The significant decreases in intracellular POD and SOD activities at high DMSO content (2% (v/v)) at 20 h (Table 1) might be ascribed to the inhibition of the synthesis of enzyme proteins by DMSO.

The less variations in SOD and intra/extracellular POD activities at low ethanol content (0.4% (v/v)) show that ethanol at low content had almost no effect on the cells. The significant decreases in SOD and intracellular POD activities at high ethanol content (2% (v/v)) might be attributed to the production of ROI as a result of the metabolism of ethanol by the cells, deactivating the enzymes by oxidation.

DMSO at low concentration levels (below 1% (v/v)) had almost no effect on the biomass, cell viability and protein contents (Figs. 1a, 2a and 3), indicating that the *T. cuspidata* cells could suffer DMSO to some extent. The obvious inhibition of DMSO on the biomass and less effect on cell viability at high dose (2% (v/v)) might suggest that DMSO inhibit the enzymes or gene expression relevant to proliferation. Sharma et al. [1] reported that DMSO (1.5% (v/v)) caused a reversible inhibition to tolormerase in a Burkitt lymphoma cell line but no appreciable cell cytotoxicity. The increase in extracellular proteins and decrease in intracellular

proteins at high DMSO dose (4% (v/v)) might indicate that the permeability of cell membrane was increase [24]. Therefore, DMSO at a low concentration level had almost no effects on the biological functions of the *T. cuspidata* cells.

The significant inhibition of ethanol (1% (v/v)) on the biomass (Fig. 1b) shows the severe toxicity of ethanol to cells, implying that the ROI and acetaldehyde produced as a result of ethanol metabolism altered the normal structure of cells due to the impaired cell defense and oxidative damage [25].

The sharp decrease in cell viability at high ethanol concentrations (over 2% (v/v); Fig. 2b) might be ascribed to the toxicity of ROI resulting from ethanol metabolism [26]. It is generally recognized that the electron transport chain of mitochondria is the major intracellular source of ROI [27]. Under normal physiological conditions, mitochondria contain sufficient antioxidants to prevent them from oxidative damages. When ethanol was added at a high dose, the ROI produced inhibited the antioxidant enzymes, impaired the cell active defense ability and perturbed the antioxidant compounds. When the ROI produced exceeded the elimination capacity of the cell defense system, the oxidative damage occurred. ROI and acetaldehyde react with proteins, lipids and nucleic acids, leading to the alterations of mitochondrial structure and functions, membrane structure and consequently the depletion of ATP [28]. As the ATP level is a reflection of cell viability [29], the loss of cell viability at higher ethanol dose (over 2% (v/v)) might show the complete depletion of ATP. The DNA random degradation (Fig. 5) occurred at the ethanol content of 2% (v/v) might indicate that ROI directly interacted with DNA leading to a severe injury to the cells.

In conclusion, ethanol below 0.4% (v/v) is suitable for monitoring the short-term effects of taxol and other water-insoluble elicitors on the suspension cultures of *T. cuspidata*, while DMSO below 1% (v/v) is appropriate for investigating the effects of water-insoluble elicitors at a long-term contact.

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