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The *ex vitro* Survival and Growth of Ginger (*Zingiber officinale* Roec.) Influence by *in vitro* Acclimatization under High Relative Humidity and CO$_2$ Enrichment Conditions

1Suriyan Cha-um, 2Nguyen Minh Tuan, 3Kongchay Phimmakong and 1Chalermpol Kirdmanee
1National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, 113 Thailand Science Park, Paholyothin Rd., Klongluang, Pathumthani 12120, Thailand
2Plant Cell Technology Laboratory, Institute of Tropical Biology, 01 Mac Dinh Chi Street, District 1, Ho Chi Minh City, Vietnam
3The National University of Laos, Department of Biology, Faculty of Science, Dongdok, Laos

**Abstract:** The aim of this investigation was to acclimatize the ginger (*Zingiber officinale* Roec.) plantlets cultured phototrophically *in vitro* under different RH and CO$_2$ concentrations and subsequent *ex vitro* adaptation. Plantlets acclimatized *in vitro* under medium or high RH (80±5 or 95±5% RH) with 1,000±100 μmol mol$^{-1}$ (CO$_2$-enrichment) conditions possessed significantly higher Relative Water Content (RWC) than those acclimatized *in vitro* under low RH (65±5% RH) with 450±100 μmol mol$^{-1}$ (CO$_2$ non-enrichment) conditions. The RWC of acclimated plantlets was positively related to maximum quantum yield of PSII photochemistry (F$_{v}$/F$_{m}$) (r = 0.77), quantum efficiency of PSII (Φ$_{PSII}$) (r = 0.89) and stomatal conductance (G) (r = 0.99), while negatively related to transpiration rate (r = 0.98). Moreover, the total chlorophyll concentration was closely related to Φ$_{PSII}$ (r = 0.77), leading to increase net photosynthetic rate (NPR) (r = 0.87). As well as, the intracellular CO$_2$ concentration (C$_{i}$) and WUE of acclimatized plantlet positively related to NPR (r = 0.96 and r = 0.85, respectively), resulting in growth promotion (r = 0.99), as defined by the parameters of leaf area, fresh weight, dry weight, root number and root length. Five days after transplantation, the WUE, C$_{i}$, F$_{v}$/F$_{m}$ and Φ$_{PSII}$ of ginger plantlets acclimatized *in vitro* under high RH with CO$_2$-enriched conditions were significantly higher than those acclimatized *in vitro* under low RH and without CO$_2$ enrichment, while E and transpiration ratio (Tr) were significantly lower. The plantlets acclimatized under high RH with CO$_2$-enrichment conditions showed the highest adaptive abilities and WUE, resulting in the highest survival percentage (90-100%) after transplantation to *ex vitro*.

**Key words:** Chlorophyll concentration, net photosynthetic rate, relative water content, survival percentage, vigorous plantlet, water use efficiency

**INTRODUCTION**

Ginger (*Zingiber officinale* Roec.) is an annual crop belonging to the family Zingiberaceae. In addition to its common usage as a spice, ginger rhizomes have a long history of use as a medicine throughout parts of Asia for its purported antiemetic and anti-inflammatory properties. Unfortunately, ginger is susceptible to soil borne diseases, most notably soft rot (caused by *Pythium aphanidermatum*) and bacterial wilt (caused by *Ralstonia solanacearum*). One of the most practical and efficient ways to solve this problem is to produce disease-free ginger from meristem-tip culture$^{[6,7]}$. Nevertheless, use of disease-free ginger from *in vitro* propagation is still limited due to the complicated transplantation process and low survival percentage after transplantation, resulting in a high cost of production$^{[3]}$.

Although, micropropagation has many advantages over the conventional propagation for large-scale production, the technique is limited by the low growth rate and low survival percentage of plantlets after being transferred to *ex vitro* conditions$^{[9]}$. The artificial conditions during *in vitro* growth result in various morphological, anatomical and physiological disorders in plantlets, which may impair the ability of plantlets to adjust to sudden changes in environmental conditions,

**Corresponding Author:** Suriyan Cha-um, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, 113 Thailand Science Park, Paholyothin Rd., Klongluang, Pathumthani 12120, Thailand
Tel: +662 5646700 Fax: +662 5646707 E-mail: suriyane@biotec.or.th
there by leading to high rates of mortality following *ex vitro* transplantation\(^5\). The poor survival percentage of plantlets after transplantation to *ex vitro* is a critical topic of investigation in the area of plant micropropagation and thus many attempts have been reported on solving this problem\(^5\). The application of an *in vitro* acclimatization phase can increase the vigor of plantlets, resulting in a higher survival percentage after transfer to *ex vitro* conditions\(^5\).

Relative Humidity (RH) is an important environmental factor of *in vitro* acclimatization\(^6,7\). RH influences the Relative Water Content (RWC) of whole plant. The RWC, in turn, affects a number of physiological characteristics, including stomatal conductance (G), CO₂ assimilation, water oxidation and Net Photosynthetic Rate (NPR). Decreasing the RWC of plantlets progressively decreased G and limited on CO₂ assimilation, thereby leading to a reduction in NPR\(^8,9\). The NPR of *in vitro* plantlets can also be restricted by the low CO₂ concentrations within culture vessels lacking adequate gas exchange\(^10\). The NPR of *in vitro* plantlets cultured under CO₂-enriched conditions was greater than those cultured on the sugar-containing media under CO₂ non-enrichment conditions\(^11,12\). CO₂-enrichment also promotes the growth of plantlets during the *in vitro* acclimatization stage\(^13,14\). The environmental factor in term of RH and CO₂ plays an important role on vigorous plantlet production with high survival percentage after transferred to *ex vitro* conditions\(^8,14,15\). The objectiveness of this investigation is to examine the acclimatization of ginger plantlets cultured photoautotrophically *in vitro* under different RH and CO₂ concentrations. The subsequent growth of plantlets after transplantation to *ex vitro* is evaluated.

**MATERIALS AND METHODS**

*In vitro* acclimatization: Single shoots of disease-free ginger (*Zingiber officinale* Rosc.) with 2-3 fully-expanded leaves (fresh weight; 300±20 mg) were aseptically excised from micropropagated-plantlets. The shoots were cultured photoautotrophically on sugar-free MS media using vermiculite as a supporting material. All shoots were cultured for 7 day at 25±2°C ambient temperature, 65±5% RH and 100 μmol m⁻² s⁻¹ photosynthetic photon flux (PPF) with a 16 h d⁻¹ photoperiod provided by fluorescence lamps (TL 36 W/84 Cool White 3350 lm, Philips, Thailand). For each treatment, twenty glass vessels containing *in vitro* cultured plantlets were placed in a plastic chamber box (length×width×height, 32×24×18 cm). Each box contained either 1500 mL KCl, a NaCl saturated-salt solution or distilled water in order to maintain RH at 65±5, 80±5 or 95±5% RH, respectively. The number of air exchange in the chamber boxes was adjusted to 5.1±0.3 h⁻¹ by punching the side of the plastic boxes with 32 holes and replacing with gas-permeable microporous polypropylene film (0.22 μm pore size) over each hole. The chamber boxes were placed into either a Plant Growth Incubator (EYELA, Model EYELATRON FLR-301LH, Japan) for CO₂ enrichment (1,000±100 μmol mol⁻¹) or a culture room for growth under the CO₂ non-enrichment (450±100 μmol mol⁻¹) condition (Table 1). All chamber boxes were incubated at 25±2°C ambient temperature and 100 μmol m⁻² s⁻¹ PPF with 16 h d⁻¹ photoperiod for 35 days (Fig. 1). The pigment concentration, net-photosynthetic rate and growth characteristics of *in vitro* acclimatized plantlets were measured prior to *ex vitro* adaptation, as described below.

**Ex vitro adaptation:** Thirty-five days acclimatization, the plantlets were transplanted into pots containing a mixture of two parts soil and one part vermiculite. Twenty plantlets in each treatment were planted out in a glass house, at 30±2°C air temperature, 75±5% RH and 300-400 μmol m⁻² s⁻¹ PPF of natural light intensity at plant level with 10 h d⁻¹ photoperiod (Fig. 1). All plants were watered twice a day. Maximum quantum yield (Fₜ/Fₘ), quantum efficiency of photosystem II (Φₚₛₚ₂), stomatal conductance (G), transpiration rate (E), transpiration ratio (Tr) and Water Use Efficiency (WUE) were measured 5 days after transfer to *ex vitro* conditions.

<table>
<thead>
<tr>
<th>Table 1: Description of treatment codes in the experiment</th>
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<tbody>
<tr>
<td>Treatment codes</td>
</tr>
<tr>
<td>LL</td>
</tr>
<tr>
<td>LM</td>
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<tr>
<td>LH</td>
</tr>
<tr>
<td>HL</td>
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<tr>
<td>HM</td>
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<td>HH</td>
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</table>

**Measurement of growth and physiological characteristics**

**Growth characteristics:** Leaf area, fresh weight, dry weight, number of root and root length of plantlets were analyzed following by Lutt method\(^16\).

**Physiological characteristics:** Transpiration rate (E, mol m⁻² s⁻¹), stomatal conductance (G, mol H₂O m⁻² s⁻¹) and transpiration ratio (Tr) were measured by the Infrared Gas Analyzer (IRGA, Model Portable Photosynthesis System Li 6400, LI-COR Inc, USA) and calculated by the Pan equation\(^17\). Water Use Efficiency (WUE) of leaves was calculated by the ratio of NPR to E according to Estrada-Luna\(^18\).

The photosynthetic systems, pigment concentration, chlorophyll a fluorescence and net photosynthetic rate, were measured. The pigment concentrations
(chlorophyll a, chlorophyll b and total carotenoid) were analyzed by the methods of Shabala[9] and Lichtenhaker[10]. The maximum quantum yield of PSII photochemistry ($F_{v}/F_{m}$) and quantum efficiency of PSII ($\Phi_{PSII}$) of the adaxial leaf surface were measured by Fluorescence Monitoring System (FMS 2; Hansatech Instruments Ltd., UK) in the pulse amplitude modulation mode, as previously described by Loggino[11]. Carbon dioxide concentration inside and outside the chamber was measured by Gas Chromatography (GC, Model GC-17A, Shimadzu Co. Ltd., Japan) and the NPR of in vitro plantlets were calculated according to Fujimori[12], while the NPR of ex vitro plantlets were measured by IRGA and then were calculated as described by Parf[13].

**Experimental design:** The experiment was designed as 2x3 factorials in a Completely Randomized Design with 4 replications and 5 plantlets per replication. Means of each treatment were compared by SPSS software (SPSS Inc., USA). The correlation between RWC and $F_{v}/F_{m}$, RWC and $\Phi_{PSII}$, RWC and G, RWC and E, chlorophyll concentration and $\Phi_{PSII}$, $\Phi_{PSII}$ and NPR, C, and NPR, WUE and NPR, NPR and dry weight, WUE and survival percentage, were evaluated by Pearson’s correlation coefficients.

**RESULTS AND DISCUSSION**

**In vitro acclimatization:** The growth characteristics (i.e. leaf area, fresh weight, dry weight, number of root and root length) of in vitro plantlets acclimatized under high RH with CO$_2$-enrichment conditions were significantly enhanced when compared with those acclimatized under low RH with CO$_2$ non-enrichment conditions (Table 2). In addition, high RH and CO$_2$-enrichment conditions significantly enhanced on the leaf area, fresh weight, dry weight, number of root and root length. The combination of these factors was also strongly affected the leaf area, fresh weight, dry weight, number of roots and root length of plantlets. Moreover, present results showed that both root length and number were greater under high RH and CO$_2$-enrichment conditions than under low RH without CO$_2$ enrichment by the factors of 2.4 and 2.8 times.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Leaf area (cm$^2$)</th>
<th>Fresh weight (mg)</th>
<th>Dry weight (mg)</th>
<th>Number of root</th>
<th>Root length (cm)</th>
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<td>73bc</td>
<td>2.8a</td>
<td>6.8bc</td>
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<td>12.1g</td>
<td>81.8</td>
<td>3.3a</td>
<td>8.8a</td>
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<tr>
<td>LH</td>
<td>2864.1</td>
<td>15.81c</td>
<td>91a</td>
<td>3.8a</td>
<td>8.8a</td>
</tr>
</tbody>
</table>

**Significant level**

Relative ** ** ** ** ** **

Table 2: Leaf area, fresh weight, dry weight, number of root and root length of ginger plantlets acclimatized in vitro under different relative humidities and CO$_2$ concentrations for 35 days.

**Table 3:** Carotenoid, chlorophyll a, chlorophyll b and total chlorophyll concentrations of ginger plantlets acclimatized in vitro under different relative humidities and CO$_2$ concentrations for 35 days.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Carotenoid (mg FW)</th>
<th>Chlorophyll a (mg FW)</th>
<th>Chlorophyll b (mg FW)</th>
<th>Total chlorophyll (mg FW)</th>
</tr>
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<tr>
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<td>1030ab</td>
<td>342ab</td>
<td>1372ab</td>
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<tr>
<td>LM</td>
<td>361a</td>
<td>1280a</td>
<td>408a</td>
<td>1688a</td>
</tr>
<tr>
<td>LH</td>
<td>339a</td>
<td>1300a</td>
<td>441a</td>
<td>1741a</td>
</tr>
</tbody>
</table>

**Significant level**

Relative ** * NS *

Table: Carotenoid, chlorophyll a, chlorophyll b and total chlorophyll concentrations of ginger plantlets acclimatized in vitro under different relative humidities and CO$_2$ concentrations for 35 days.

**NS** Significance at p<0.01 or 0.05, respectively. Means within a row followed by the different letters in each column are significantly different at p<0.01 by New Duncan’s Multiple Range Test respectively. The root system of the plantlets plays a critical role in water uptake and the translocation of many metabolites. Plantlets acclimatized under low RH, with or without CO$_2$-enrichment, exhibited the symptoms of water-deficit (i.e. chlorosis, leaf burn and growth inhibition; data not shown).

The highest Relative Water Content (RWC), 94%, was obtained when ginger plantlets acclimatized under high RH (95±5% RH) with CO$_2$-enrichment (1000±100 μmol mol$^{-1}$). The RWC of acclimatized plantlets was directly reduced to 56% by the low percentage of
Fig. 2: Correlation between relative water content and maximum quantum yield of PSII photochemistry (A) as well as relative water content and quantum efficiency of PSII (B) of ginger plantlets acclimatized in vitro under different relative humidities and CO₂ concentrations for 35 days and subsequently transferred to ex vitro conditions for 5 days.

Fig. 3: Correlation between relative water content and stomatal conductance (A) as well as relative water content and transpiration rate (B) of ginger plantlets acclimatized in vitro under different relative humidities and CO₂ concentrations for 35 days and subsequently transferred to ex vitro conditions for 5 days.

RH (65±5%RH) and without CO₂ enrichment in the culture chamber. The RWC of acclimatized-plantlets was positively related to $\Gamma_T/\Gamma_w$ ($r = 0.77$), $\Phi_{PSII}$ ($r = 0.89$) and $G$, but negatively related to $E$ ($r = 0.98$) (Fig. 2 and 3). The total carotenoid, chlorophyll a, chlorophyll b and total chlorophyll concentrations of in vitro plantlets acclimatized under high RH with CO₂-enrichment conditions were significantly higher than those acclimatized under low RH without CO₂ enrichment by factors of 2.65, 5.02, 4.28 and 4.81 times, respectively (Table 3). RH factors significantly affected on chlorophyll concentration but not carotenoid concentration, whereas CO₂-enrichment increased the concentration of carotenoid and both chlorophyll a and b. The total chlorophyll concentration of acclimatized-plantlets positively related to $\Phi_{PSII}$ ($r = 0.77$) (Fig. 4A), which is consistent with the role of chlorophylls as the light harvesting complexes of the photosynthetic system. The $\Phi_{PSII}$ of plantlets acclimatized under high RH with CO₂-enrichment conditions was 1.38 times higher than those acclimatized under low RH with CO₂ non-enrichment conditions. The decreasing of this parameter was associated with a reduction in NPR ($r = 0.87$) (Fig. 4B).

The stomata function (conductance) of acclimatized plantlets was strongly stimulated by the high RH and CO₂-enrichment conditions, defined by high G parameter (Fig. 3A). Normally, the stomata pores of plantlets under high RH and CO₂-enrichment are widely opened for CO₂-fixation. On the other hand, the stomata of plantlets under low RH or water-deficit condition are less opened, or even remain closed to prevent water loss (Fig. 3B). In addition, the intracellular CO₂ ($C_i$) and WUE of
acclimatized-plantlets under high RH and CO₂-enrichment conditions were significantly higher than those acclimatized under low RH with CO₂ non-enrichment conditions. The Ci and WUE parameters of acclimatized-plantlets closely related to NPR (r = 0.96 and r = 0.85, respectively) (Fig. 5). The NPR of acclimatized-plantlets under high RH with CO₂-enrichment conditions was enhanced more than those acclimatized under low RH without CO₂ enrichment. This parameter positively related to dry weight or growth characteristics of acclimatized-plantlets (r = 0.99) (Fig. 6A).

The ideal RH for in vitro plantlets acclimatization is dependent upon the plant species and the transferring procedures used[13,39]. However, ginger plantlets acclimatized under low RH exhibited low RWC or water deficit. Reducing RH conditions during acclimatization results in better adaptation to ex vitro conditions in the cases of potato [14], Eucalyptus [9] and Thai neem [22]. Low RWC in plantlets has a negative effect on both the dark reaction of photosynthesis [i.e. low stomatal conductance (G), low CO₂-assimilation and high transpiration rate (E)] and light reaction [i.e. low F/Fₙ and low Φₚₙ], resulting in an overall reduction in NPR [9,11,23]. On the other hand, the high RWC of ginger plantlets acclimatized under high RH with CO₂-enrichment conditions prevented on pigment degradation (Table 3), resulting in stimulation of light harvesting for water oxidation or ATP production in the light reaction, as represented by the regulation of chlorophyll a fluorescence (i.e. F/Fₙ and Φₚₙ), as well as regulation of stomata for CO₂-assimilation [16,27]. The photosystem II regulation and CO₂-assimilation of ginger plantlets acclimatized under high RH with CO₂-enrichment conditions were significantly higher than those acclimatized under low RH with CO₂ non-enrichment conditions. The Ci and WUE parameters of acclimatized-plantlets closely related to NPR (r = 0.96 and r = 0.85, respectively) (Fig. 5). The NPR of acclimatized-plantlets under high RH with CO₂-enrichment conditions was enhanced more than those acclimatized under low RH without CO₂ enrichment. This parameter positively related to dry weight or growth characteristics of acclimatized-plantlets (r = 0.99) (Fig. 6A).

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Fig. 6: Correlation between NPR and dry weight (A) as well as water use efficiency and survival percentage (B) of ginger plantlets acclimatized in vitro under different relative humidities and CO₂ concentrations for 35 days and subsequently transferred to ex vitro conditions for 5 days.

Table 4: Water use efficiency, stomatal conductance, transpiration rate, transpiration ratio, maximum quantum yield of PSII photochemistry and quantum efficiency of PSII of ginger plantlets acclimatized in vitro under different relative humidities and CO₂ concentrations for 35 days and subsequently transferred to ex vitro conditions for 5 days.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Water use efficiency (%)</th>
<th>Stomatal conductance (μmol H₂O m⁻² s⁻¹)</th>
<th>Transpiration rate (mmol m⁻² s⁻¹)</th>
<th>Transpiration ratio</th>
<th>Maximum quantum yield of PSII</th>
<th>Quantum efficiency of PSII</th>
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</thead>
<tbody>
<tr>
<td>LL</td>
<td>0.6c</td>
<td>13.2c</td>
<td>0.35a</td>
<td>6.72a</td>
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<td>LM</td>
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<td>0.24b</td>
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<td>0.78a</td>
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<td>HH</td>
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<td>0.14c</td>
<td>0.28d</td>
<td>0.80a</td>
<td>0.76a</td>
</tr>
</tbody>
</table>

Means within a row followed by different letters in each column are significantly different at p<0.01 by New Duncan's Multiple Range Test.

Directly enhanced NPR and growth. In photoautotrophic system, CO₂ in the culture vessel is the main carbon source for photosynthesis. Normally, the CO₂ inside of sealed culture vessels is quite low due to the limited rate of the gas exchange. Thus, the elevation of CO₂ in a photoautotrophic system is an alternative method for NPR promotion. CO₂-enrichment of in vitro plantlets directly enhanced CO₂-uptake by increasing stomatal conductance or stoma-opening for CO₂-assimilation, resulting in the high C₅¹ for photosynthesis system.

**Ex vitro adaptation:** The WUE, G, E, Tr, F_v/F_m and Φ_PN were measured after ex vitro adaptation. Plantlets acclimatized under high RH with CO₂-enrichment conditions were better adapted to ex vitro conditions than those acclimatized under low RH with CO₂ non-enrichment conditions. The WUE and G of ex vitro plantlets were enhanced when acclimatized under high RH with CO₂-enrichment conditions, while E was decreased. The WUE of acclimatized plantlets was positively related to survival percentage (r = 0.90) (Fig. 6B). In addition, chlorophyll a fluorescence as determined by F_v/F_m and Φ_PN, the energy production of photosystem II was highest for plantlets acclimatized under high RH with CO₂-enrichment conditions (Table 4). Present results indicate that plantlets acclimatized under high RH with CO₂-enrichment conditions possess vigorous root systems, a high efficiency of water relation and a high NPR consistent with vigorous plantlets. These plantlets displayed the highest survival percentage when transplanted to ex vitro conditions.

The physiological adaptations of vigorous plantlets with high survival percentage in ex vitro conditions have been widely investigated in many plant species i.e. pepper¹⁰, tobacco¹¹, carnation¹², neem tree¹³ and Eucalyptus¹⁴. Ginger plantlets acclimatized under high RH with CO₂-enrichment conditions grew vigorously in vitro and rapidly adapted to ex vitro conditions. These
plantelets increased CO₂ assimilation through higher WUE and G, while reducing water-loss through decreases in E and Tr, most likely through the regulation of stomata. Furthermore, these plantlets possessed the highest water oxidation, Fₚ/Fₘ and ϕ₁₇₂ after transplant to ex vitro, as well as the high survival percentage. The Fₚ/Fₘ and ϕ₁₇₂ parameters have been used as an indicator of water-deficit damages of PSII reaction center, as well as of physiological adaptation after transfer to ex vitro conditions.

In conclusion, in vitro acclimatization of ginger plantlets under high RH with CO₂-enrichment produced plantlets with both vigorous shoot and root systems of the various combinations of RH and CO₂ concentration tested, they exhibited the highest water use efficiency, pigment concentration and water oxidation in PSII, leading to the highest NPR. These plantlets rapidly adapted to ex vitro conditions and demonstrated by their enhanced growth and high survival percentage.

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