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Somatic Cell Differentiation and Rapid Clonal Propagation of Aspen

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Summary

Tissue culture*) techniques were employed for investigating growth and differentiation in relation to rapid clonal propagation of difficult to root aspen *(Populus)* genotypes. Bud stem, leaf and root explants from 48 aspen clones, including *P. tremula, P. tremuloides* and their hybrids, were cultured on modified Woody Plant Medium (WPM), here designated Aspen Culture Medium (ACM). Results of the present study indicate that bud explants can be induced to undergo growth and organogenesis, without much visible callus formation. So far a large number of plants have been regenerated via tissue culture technology from 8 *P. tremula* clones and 6 hybrid aspen *(P. tremula* × *P. tremuloides)* clones, and from these several hundred plants have been transplanted under field conditions. Plantlet regeneration in 8 more clones is in progress. However, many aspen clones (26 of 48) were largely unresponsive to the *in vitro* conditions provided, indicating that growth and differentiation response is not only controlled by the cultural environment, but is also dependent upon the genotype. The potential of root, leaf, and stem tissues for rapid clonal propagation is being further explored.

Key words: Aspen *(Populus)*, Hybrid aspen, Somatic cell differentiation, Clonal propagation, Clonal variation, Tissue culture.

Zusammenfassung

Gewebekulturtechniken wurden für die Untersuchungen des Wachstums und der Differenzierung im Zusammenhang mit der schnellen klonalen Vermehrung von Genotypen der Aspe, einer bei vegetativer Vermehrung schwer zu bewurzelnden Baumart, angewandt. Explantate, die von der Knospe, dem Stamm, dem Blatt und der Wurzel entnommen wurden, von insgesamt 48 Aspenklonen der beiden reinen Arten *Populus tremula* und *Populus tremuloides sowie* deren Hybriden wurden auf einem modifizierten Woody Plant Medium, das hier als Aspen Culture Medium bezeichnet wird, kultiviert. Die Ergebnisse der vorliegenden Untersuchung zeigen, das Knospenexplantate zu Wachstum und Organogenese angeregt werden können, ohne viel sichtbaren Callus zu bilden. Bislang wurden mehr als 1000 Pflanzen mit Hilfe der Gewebekulturtechnik regeneriert, und zwar von acht Klonen *(Populus tremula)* und sechs Hybridaspen-Klonen *(Populus tremula* × *Populus tremuloides).* Hiervon wurden bereits mehrere 100 Pflanzen unter Feldbedingungen aufgezogen. Die Regeneration von kleinen Pflanzen macht bei weiteren acht Klonen Fortschritte. Viele Aspenklone (26 von 48) zeigten jedoch weitgehend keine Reaktion auf die gegebenen in vitro-Bedingungen. Dies zeigt an, daß Wachstum und Differenzierung nicht nur durch die Kulturbedingungen kontrolliert werden, sondern auch von der Reaktion des Genotyps abhängig sind. Das Potential der Wurzel, Blatt- und Stammexplantate, die für eine schnelle klonale Vermehrung verwendet werden, wird weiter erforscht.

Introduction

Tissue explants from tree species are generally difficult to grow and differentiate *in vitro.* This is further compounded by the fact that presence of genetic variation between members of a tree species makes it extemely difficult to design an allpurpose medium for the growth and differentiation of tissues from a given tree species. Nevertheless, slow but gradual progress has been made, in the last few years, towards culture of organs, tissues, and protoplasts from woody plants (see reviews by Karnosky 1981; David 1982; Brown and Sommer 1982; Ahuja 1982). At the present time, a good deal of effort is directed towards vegetative propagation of tree species through tissue culture technology, In many tree species woody cuttings are difficult to root, and in others the rooting frequency may be rather low. In such cases, tissue culture techniques may be exploited as means of accomplishing clonal propagation. Aspen *(Populus* species) are difficult to root from woody cuttings. Previous tissue culture studies based on callus and bud explants of aspen *(P. tremula* and *P. tremuloides)* have revealed that it is possible to induce differentiation of roots, shoots, and plantlets under *in vitro*

*) The term tissue culture is used in the broadest sense to include culture of meristematic explants from buds, leaf discs, stem segments, and root segments.

conditions (Wolter 1968; Winton 1970, 1971; Chalupa 1974; Christie 1978).

We have been interested in rapid clonal propagation of a large number of selected aspen genotypes, including clones from European aspen *(P. tremula),* quacking aspen *(P. tremuloides)* and their hybrids. Our preliminary tissue culture studies (Ahuja and Muhs 1982) based on bud explants of a few aspen genotypes have indicated differences in their regenerative abilities. The present investigation deals with growth and differentiation response of different somatic tissues, including bud, stem, leaf, and root explants, from a large number of aspen clones, majority of which not previously investigated, and relevance of these morphogenetic studies to rapid clonal propagation.

Materials and Methods

Tissue from bud, stem, leaf and root explant of aspen were cultured under aseptic conditions on modified Murashige and Skoog's (1962) medium (MSM) and woody plant medium (WPM) developed by Lloyd and McCown (1981). The modified form of WPM, which promoted good growth and differentiation of aspen tissues, is in this paper designated Aspen Culture Medium (ACM; *Table 1).* A total of 48 aspen clones derived from 2n, 3n and 4n *Populus tremula,* 2n *P. tremuloides,* and their 2n and 3n hybrids were included in the present study *(Table 2).* Tissue explants from established cuttings in the nursery as well as trees (ages 17 to 40 years) were cultured to study growth and differentiation response. Before culturing, tissues were surface sterilized in 3—5% sodium hypochlorite for 5—15 minutes, and subsequently washed three times in double distilled autoclaved water. From the buds, only the meristematic part was cultured; from the leaves, small (8—10 mm) leaf discs or small young leaves; from stems, only parenchyma along with vascular bundle; and from roots, cut segments of various lengths were cultured. Cultures were maintained at 25° C ± 2° C, 70% relative humidity and a 16 hours photoperiod (2000—4500 lux), or in total darkness.

Table 1. — Comparison of basal Murashige and Skoogs' medium (MSM), Lloyd and McCown's Woody Plant Medium (WPM), and the Aspen Culture Medium (ACM) employed in the present study.

	Concentrations in mg/l		
	MSM	WPM	ACM
A. Macronutrients			
NH_4NO_3	1650	400	400
KNO_3	1900	-	-
$CaNO_3 \cdot 4H_2O$	-	556	556
K_2SO_4	-	990	990
$CaCl_2 \cdot 2H_2O$	440	96	96
$MgSO_4 \cdot 7H_2O$	370	370	370
KH_2PO_4	170	170	170
B. Iron			
$Na_2 \cdot EDTA$	37.25	37.3	-
$FeSO_4 \cdot 7H_2O$	27.85	27.8	-
Sodium Ferric·EDTA	-	-	30
C. Micronutrients			
$MnSO_4 \cdot H_2O$	22.3	22.3	22.3
$ZnSO_4 \cdot 7H_2O$	8.6	8.6	8.6
H_3BO_3	6.2	6.2	6.2
KI	0.83	-	0.83
$Na_2MOO_4 \cdot 2H_2O$	0.25	0.25	0.25
$CuSO_4 \cdot 5H_2O$	0.025	0.25	0.025
$CoCl_2 \cdot 6H_2O$	0.025	-	0.025
D. Vitamins			
Thiamine · Hcl	0.1	1.0	0.1
Nicotinic acid	0.5	0.5	0.5
Pyridoxine · Hcl	0.5	0.5	0.5
Glycine	2.0	2.0	-
Lysine	-	-	100
E. Sugars			
Myo-inositol	100	100	100
Sucrose	30.000	20.000	20.000
pH	5.7 - 5.8	5.2	5.5 - 5.6

Table 2. — Data obtained from *in vitro* shoot growth and differentiation studies from bud explants of 48 aspen clones.

Species or hybrids	No. of clones tested	No. of clones that grew well	No. of clones that grew slowly	No. of clones that grew poorly or not at all
2n *P. tremula*	9	5	4	
3n *P. tremula*	1			1
4n *P. tremula*	1			1
2n *P. tremuloides*	4			4
2n Hybrid aspen (*P. tremula* X *P. tremuloides*)	31	4	8	19
3n hybrid aspen (*P. tremula* X *P. tremuloides*)	2	1		1
Total	48	10	12	26

Results and Discussion

Since aspen tissues showed a better growth response on modified WPM (here designated ACM) as compared to MSM under our experimental conditions, we carried our growth and organogenesis experiments on ACM. Previous studies on *in vitro* growth and morphogenesis in aspens were based on modified Wolter and Skoogs (1966) medium (Wolter 1968; Winton 1970, Chalupa (1974) and modified MSM (Christie 1978).

Bud Explants

Bud explants from a number of aspen genotypes differentiated shoots when cultured on ACM fortified with 20 mg/l adenine sulfate and 0.4—0.5 mg/l 6-benzylaminopurine (BAP) and kept under a light regime of 2000—3000

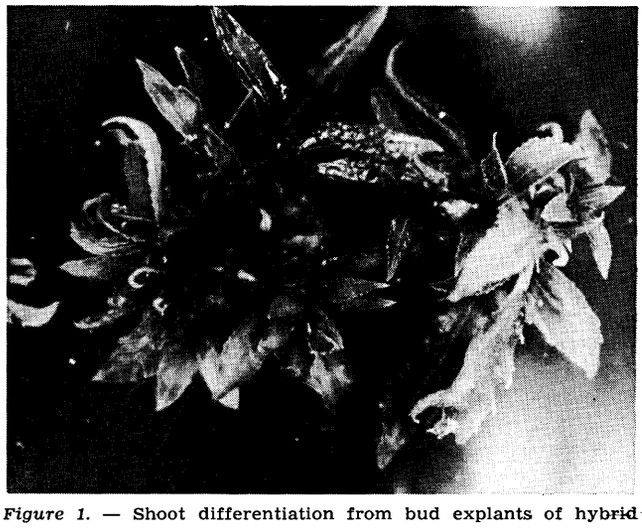

Figure 1. — Shoot differentiation from bud explants of hybrid aspen (*P. tremula* × *P. tremuloides*).

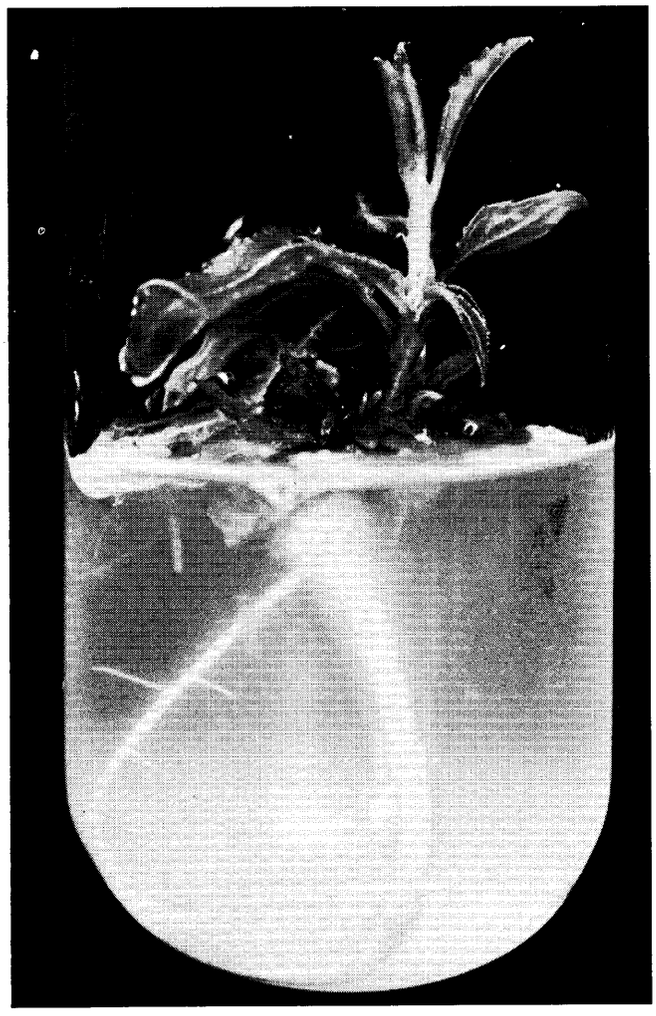

Figure 2. — Plantlet formation following culture of bud-derived shoot of hybrid aspen.

lux. Three to four weeks later, these tissues were transfered to the above ACM containing, in addition, low concentration (0.02 mg/l) of naphthaleneacetic acid (NAA). On this medium further shoot proliferation occurred *(Figure 1)*. Generally, bud explants from the nursery grown aspens showed a better growth response than buds taken from older trees. However, we have obtained growth and differentiation of shoots from the bud explants of several older aspen trees (upto 40 years of age), and have regenerated plantlets from these shoots.

In our experiments with differentiation of shoots from the bud explants, there was very little visible callus formation. We emphasize this point. We believe that extensive callus formation and longterm callus culture may be accompanied by variability under cultural conditions (AHUJA 1982). For mass cloning of selected genotypes, it is necessary to keep culture-induced genetic variability at its lowest or an acceptable level.

After 6—8 weeks (sometimes longer) of bud explant culture, a constant supply of shoots can be regularly harvested, from each bud for a long time, for plantlet regeneration. The shoots derived from bud explanted differentiated roots *(Figure 2)* on the rooting ACM lacking myoinositol and lysine, but containing low levels of auxin, such as indolebutyeric acid (IBA; 0.5 mg/l) and NAA (0.1 mg/l). Rooting under our cultural conditions (4000—4500 lux) occurred in 8—10 days on the bud derived shoots. So far more than a thousand plants have been regenerated, by employing tissue culture technology, from 8 *P. tremula* clones (including 5 from trees of ages 25—40 years) and 6 hybrid aspen clones (including 2 from trees of ages 17 and 23 years). The plants derived from tissue culture were hardened in decreasing levels of humidity in our controlled chambers and greenhouse *(Figure 3)* before planting them out under the field conditions. Barocka (see FRÖHLICH 1982) has employed bud explant method for mass cloning of a triploid aspen clone, A s t r i a . From our initial experiments started in October 1981, we have already carried several hundred tissue culture derived plants from two *P. tremula* and two hybrid aspen clones in the field conditions *(Figure 4)* to test their uniformity and the growth performance under field conditions.

Stem, Leaf and Root Explants

Stem explants from a few *P. tremula* and hybrid aspen clones tested formed calli when grown ACM supplemented with NAA (1.0—2.0 mg/l) and BAP (0.1 mg/l), and kept under total darkness. By manipulating these hormones, it was possible to induce shoots, roots or both on the stem callus following growth under 16 hours photoperiod (3000 lux). In one *P. tremula* clone, we have regenerated plants from the shoots derived from the stem callus. Tissue culture plants regenerated from callus have been previously reported in *P. tremula* (WINTON 1971; CHALUPA 1974), *P. tre-*

Figure 3. — Tissue culture derived aspen plants under greenhouse conditions.

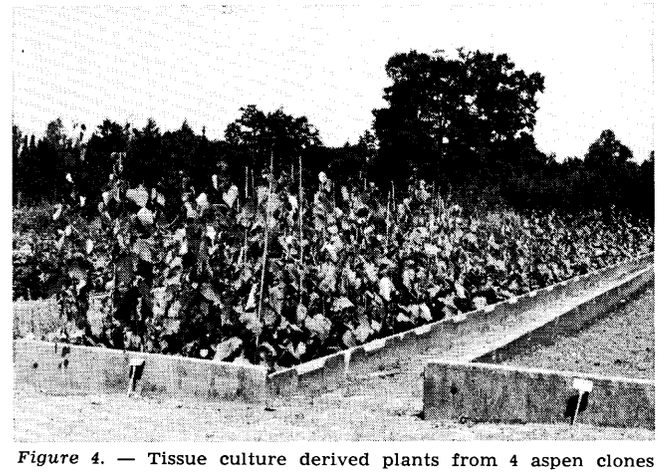

Figure 4. — Tissue culture derived plants from 4 aspen clones (2 *P. tremula* and 2 hybrid aspen) under field conditions.

muloides (WOLTER 1968; WINTON 1970). Although Callus culture method has potential for microvegetative propagation of plants, we have not used this route for clonal propagation, but intend to explore this avenue for searching somaclonal variation (LARKIN and SCOWCROFT 1981).

In addition to stem explants, we have also cultured leaf discs and root explants of aspen. Leaf discs/small leaves can be induced to differentiate shoots when cultured on an appropriate cytokinin/auxin (0.5 mg/l BAP + 0.02 mg/l NAA) medium *(Figure 5)*. We have regenerated plants from leaf disc-derived shoots in one hybrid aspen clone, and intend to explore this approach further for clonal propagation of different aspen genotypes. Leaf disc approach also offers opportunities for monitoring somaclonal variation in aspen, since questions have been raised regarding the genetic constitution of leaf cells (see SHEPARD *et al.* 1980). We have also regenerated shoots from root explants of *P. tremula (3* clones) and hybrid aspen (4 clones) on ACM containing BAP (0.5 mg/l) and NAA (0.02 mg/l). In all these clones shoots differentiated almost directly (without visible callus formation) from the root explants *(Figure 6)*, Since aspens are known to sprout shoots from root suckers in nature or under nursery conditions, we believe root explants may also be employed for microvegetative propagation.

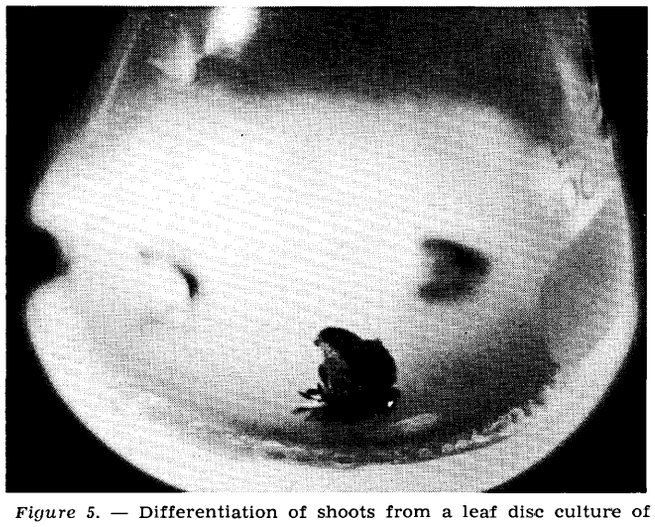

Figure 5. — Differentiation of shoots from a leaf disc culture of hybrid aspen.

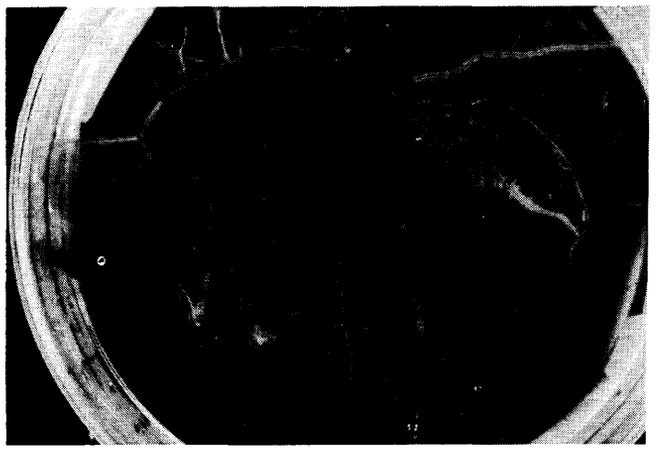

Figure 6. — Formation of shoots from root explant cultures of aspen.

Figure 7. — Growth and differentiation on bud explants of *P. tremuloides* after 35 days in culture. Note callus and some shoot formation.

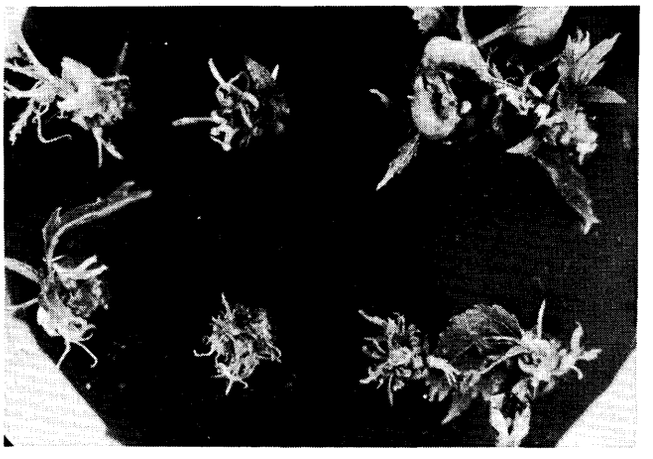

Figure 8. — Growth and differentiation on bud explants of *P. tremula* after 26 days in culture. Note extensive shoot differentiation and minimal callus formation.

Clonal Variation

Genetic variation as measured by morphogenetic response was evident between different aspen species as well as between the clones of the parental species, and between hybrid aspen clones. Bud explants from different clones showed different growth and differentiation response when cultured on a common ACM. Bud explants from *P. tremuloides* grew very poorly and developed mainly small callus but very few shoots *(Figure 7)*. On the other hand, bud explants from *P. tremula* and hybrid aspen, in general, differentiated many shoots without much callus formation *(Figure 8)*. However, bud explants from different clones of *P. tremula* and hybrid aspen did not all grow at the same rate, and a number of clones grew/differentiated very little or not at all. *Table 2* summarizes the results of shoot regeneration from bud explants of 48 clones in aspen.

There were also differences between clones with regard to ability to differentiate roots *in vitro*. For example, when shoots from the bud explants of hybrid aspen clone were cultured on a rooting ACM, they invariably formed callus first at the cut end of shoots, and then differentiated roots from the callus *(Figure 9)*. On the other hand, bud derived shoots from another hybrid aspen clone differentiated roots

directly, without much visible callus formation, from the cut end of the shoots *(Figure 10)*. In a third hybrid aspen clone, bud derived shoots did not form callus or differentiate roots when cultured on a common rooting ACM. Aspen genotypes also responded differently when exposed to low levels of antibiotics (Penicillin/streptomycin solution; Serva). In a series of experiment, we dipped the cut ends of the tissue culture derived shoots from bud explants in 1/10 dilution of the antibiotic solution for protection against infection before transfering them to the rooting ACM. To our surprise, the rooting process was hastened by a short treatment with antibiotics in most aspen genotypes: 5—7 days against 8—10 days without antibiotic treatment. However, one hybrid aspen clone showed sensitivity to antibiotic treatment: the cut end of the shoot turned black as if it were burned and failed to form callus or differentiate roots. In addition to genetic variation between clones, in terms of their regenerating abilities, somaclonal variation was also observed in some cultures. Variegated plants and differences in the morphology of roots were observed. Stubby as well as short secondary roots were observed in *P. tremula* cultures.

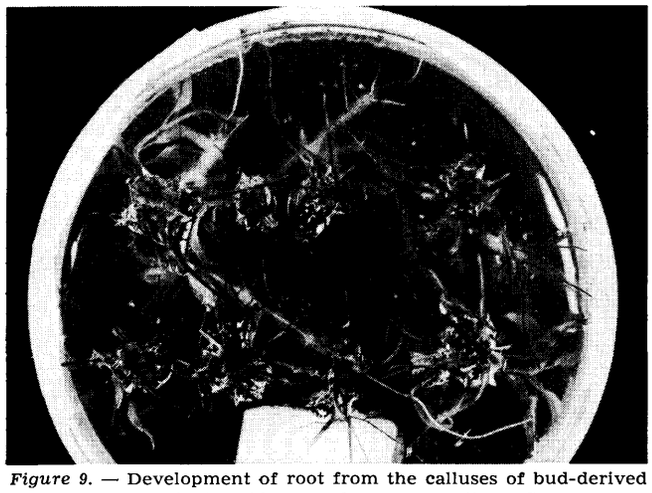

Figure 9. — Development of root from the calluses of bud-derived shoots of a hybrid aspen clone on a rooting ACM.

Figure 10. — Development of roots almost directly from the bud-derived shoots of a hybrid aspen clone on a rooting ACM. Note minimal visible callus formation at the point of origin of roots.

Concluding Statement

Tissue explants from different parts of aspen (bud, stem, leaf, and root) can be manipulated under *in vitro* conditions to undergo organogenesis. Plantlet regeneration from cultured explants has been accomplished in a number of aspen clones. All the aspen clones tested did not respond the same way to the *in vitro* conditions *provided:* 22 of 48 clones exhibited growth and differentiation, while the remaining 26 showed little or no growth, indicating differences between regenerative capacities of different aspen clones. For regeneration studies on the unresponsive clones, further investigations are necessary on the cultural conditions, effect(s) of site or location on a clone, and the best time of the year for the culture of tissue explants.

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