

# ISOLATING GENE(S) CONFERRING RESISTANCE/SUSCEPTIBILITY TO INFECTION OF *Corynespora cassicola* IN *Hevea brasiliensis*

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Rubber tree (*Hevea brasiliensis* Muell Arg.) is an important commodity crop in Malaysia. It belongs to the family Euphorbiaceae and is planted for its latex and timber. It is widely exposed to a multiple of disease which includes leaf, bark and roots. The identification of genes whose expression is altered under conditions of stress is an important first step towards understanding the response of this species. Four selected *Hevea* clones at the two-whorls stage were inoculated with 2000 spores/ml and left to incubate for 3 days in an environmentally-controlled chamber. Changes in gene expression in their leaves were detected via differential display reverse transcriptase PCR (DDRT-PCR). The identified cDNAs were from Populus EST from severe drought-stressed leaves.

*Corynespora* Leaf Fall (CLF) is a major leaf disease affecting *Hevea* in Malaysia. This disease occurs all year round and affects both mature and immature leaves. The severity of the disease is influenced partly due to the susceptibility of selected rubber clones. In a susceptible clone, infection with *C. cassicola* will result in complete defoliation and becomes stunted (Chee, 1990). The symptoms vary with isolates of the pathogen as demonstrated by *C. cassicola* infections in Sri Lanka and Malaysia (Chee, 1988). It has been shown that different races exist within *C. cassicola* and they are classified as Race 1 and Race 2 (Ismail and Jeyanangi, 2000; Safiah and Noor Hisham, 2003). Race 1 of the pathogen was found to infect the earlier *Hevea* clones, e.g. RRIM 600, and Race 2 infects the newer clones, e.g. RRIM 2020.

Differential display was first described as a way to identify and clone eukaryotic, and later to differentially expressed genes (Liang and Pardee, 1992). The main advantage of this method is its ability to display rapidly and simultaneously the expression of mRNA from variant cells having the same genetic background. As it is PCR-based, it has higher sensitivity which is particularly useful for analysis of systems that have limiting biological materials such as studying the differential gene expression of fungal-pathogen in plants during the early stages of the infection process (Zimmermann, 1994; Bradshaw *et al.*, 2006). Differently expressed genes are then cloned and subsequently sequenced and used as primers or probes to isolate the genes of interest.

This paper will discuss the use of DDRT-PCR technique to identify differentially expressed cDNA that are present in susceptible and tolerant *Hevea* plants and to identify cDNA using the NCBI BLAST database.

## MATERIALS AND METHODS

### Plant Materials

*Hevea* plants were selected from the family PB 5/51 x IAN 873 (Table 1) after undergoing disease screening with *C. cassiicola* Race 2 (CLN16). The most susceptible (mean disease score between 2.5 to 5.0) and least susceptible (mean disease score between 0 and 2.4) were selected for this experiment.

Least Susceptible		Susceptible	
Plant	Mean disease score	Plant	Mean disease score
KT 34/25	0.30	KT13/50	3.30
KT 31/24	0.50	KT 14/48	2.40

**Table 1.** KT clones used for the differential display experiment and their mean disease score when challenged with isolate CLN 16.

### Inoculation of plants in polybags

Selected plants at the two-whorl stage were used in this experiment. Inoculums from *C. cassiicola* isolate CLN 16 with the concentration of 2,000 spores/ml were sprayed onto the abaxial surface of the young leaves (Fig 1). The whole plantlet was covered in a plastic bag along with wet cotton balls to keep the environment within the bag moist (Fig 2). The moist condition is necessary to ensure infection of the fungus on the leaves. The bags were removed after 24 h (Fig 3). The chamber was kept humid by periodical misting with water and the temperature maintained at 24°C. Leaves were sampled before spraying the plants with the spores (0 h) and again at three days (72 h) after inoculation.



**Fig 1:** Plants were sprayed with spores (2,000 spores/ $\mu$ l). The plant was placed within an environmentally controlled chamber.



**Fig 2:** Plants were then covered in a plastic bag to keep the environment within the bag moist.



**Fig 3:** The bags were removed after 24 h. A mister was programmed to mist the chamber to keep the environment within the chamber moist.

## **Extraction of Total RNA**

The extraction protocol used was adopted from Prescott and Martin (1987). The concentration of RNA was determined by measuring the absorbance of a sample at  $A_{260}$  in a spectrophotometer (UV/VIS Spectrometer Lambda EZ 201, Perkin Elmer). The RNA was then diluted accordingly to the concentration of 10  $\mu\text{g}/\mu\text{l}$ .

## **Differential Display Reverse Transcriptase PCR (DDRT-PCR)**

### DDRT-PCR Labelled with Fluorescence Dye

Differential display was performed using the RNAimage™ Kit (GenHunter Corporation) according to the supplier's protocol which consists of two PCR reactions. The first PCR step involves the reverse transcription of the mRNA with oligo-dT primers (H-T<sub>11</sub>M) anchored to the beginning of the poly(A) tail, followed by the second PCR reaction in the presence of a second short primer which is arbitrary in sequence (H-AP1-24) (Table 2).

Primer sets that produced polymorphic fingerprinting patterns are further labeled with radioisotopes [ $\alpha$ -P33] dATP (3000 Ci/mmol). The amplified cDNA was resolved on 6% denaturing polyacrylamide gel containing 7 M urea at constant 60 W for 3 h. This was followed by drying the gel on Whatmann 3MM without fixing and exposed to X-ray film for 7 days before developing the film with KODAK developer and fixer.

## **Isolation, Re-amplification and Cloning of cDNA Bands**

### Isolation of cDNA Bands

Differentially expressed bands, which were present in either susceptible or resistant plants, were excised from the dried gel. The autoradiogram was aligned with the dried gel and the bands of interest were cut out using a clean scalpel and the gel slice with the 3M paper was placed in a 1.5 ml microfuge tube. Distilled water (100  $\mu\text{l}$ ) was added in the microfuge tube and soaked for 10 min.

The microfuge tube was boiled for 15 min and spun for 2 min to collect the condensation and pellet the gel and paper debris. The supernatant was carefully transferred to a new 1.5 ml microfuge tube and 10  $\mu\text{l}$  of 3 M sodium acetate, 5  $\mu\text{l}$  glycogen and 450  $\mu\text{l}$  of 100% ethanol was added and incubated at  $-80^{\circ}\text{C}$  for 30 min to precipitate the DNA into the solution. The tubes were centrifuged at 14,000 rpm for 10 min at  $4^{\circ}\text{C}$  to pellet the DNA. The supernatant was removed and the pellet was rinsed with 200  $\mu\text{l}$  ice-cold 80% ethanol. The tube was spun briefly to remove residual ethanol and the pellet was air dried. The pellet was dissolved in 10  $\mu\text{l}$  distilled water.

### Re-amplification of cDNA Bands

The recovered DNA was re-amplified in a 40  $\mu\text{l}$  PCR reaction according to the protocol provided by the manufacturer (GeneHunter Corporation, USA).

Oligo-dT Primers	Arbitrary 13-mer Primers	
H-T <sub>11</sub> A: 5'-AAGCTTTTTTTTTTTA	H-AP1:	5' AAGCTTGATTGCC
H-T <sub>11</sub> C: 5' AAGCTTTTTTTTTTTC	H-AP2:	5'-AAGCTTCGACTGT
H-T <sub>11</sub> G: 5' AAGCTTTTTTTTTTTTG,	H-AP3:	5'-AAGCTTTGGTCAG
	H-AP4:	5'-AAGCTTCTCAACG
	H-AP5:	5'-AAGCTTAGTAGGC
	H-AP6:	5'-AAGCTTGCACCAT
	H-AP7:	5'-AAGCTTTTACCGC
	H-AP8:	5'-AAGCTTTTACCGC
	H-AP9:	5'-AAGCTTCATTCCG
	H-AP10:	5'-AAGCTTCCACGTA
	H-AP11:	5'-AAGCTTCGGGTAA
	H-AP12:	5'-AAGCTTGAGTGCT
	H-AP13:	5'-AAGCTTCGGCATA
	H-AP14:	5'-AAGCTTGGAGCTT
	H-AP15:	5'-AAGCTTACGCAAC
	H-AP16:	5'-AAGCTTTAGAGCG
	H-AP17:	5'-AAGCTTACCAGGT
	H-AP18:	5'-AAGCTTAGAGGCA
	H-AP19:	5'-AAGCTTATCGCTC
	H-AP20:	5'-AAGCTTGTTGTGC
	H-AP21:	5'-AAGCTTTCTCTGG
	H-AP22:	5'-AAGCTTTTGATCC
	H-AP23:	5'-AAGCTTGGCTATG
	H-AP24:	5'-AAGCTTCACTAGC

**Table 2.** Oligo-dT and 13-mer arbitrary primers used in analysis

#### Ligation and Transformation of cDNA Bands

The re-amplified cDNAs were cloned into pGEM-T vector (Promega) using the protocol provided by the manufacturer.

LB/ampicilin/IPTG/X-gal plate was prepared and 100 µl transformant was spread using a sterile glass spreader. The LB plate was then incubated at 37°C overnight (16 h).

The resultant white colonies were picked using sterile toothpicks and grown in 3 ml LB/Ampicilin in McCartney bottles.

#### Sequencing of cDNA inserts

Plasmids were purified according to Sambrook *et al.* (1989) and sent to First Base Laboratories Sdn. Bhd. for sequencing. The results were sent back via email in a Chromass file. Each sequence was compared with sequences in NCBI Basic Local Alignment Search Tool (BLAST) using translated nucleotide database using a translated nucleotide query (tblastx).

## Northern Blot

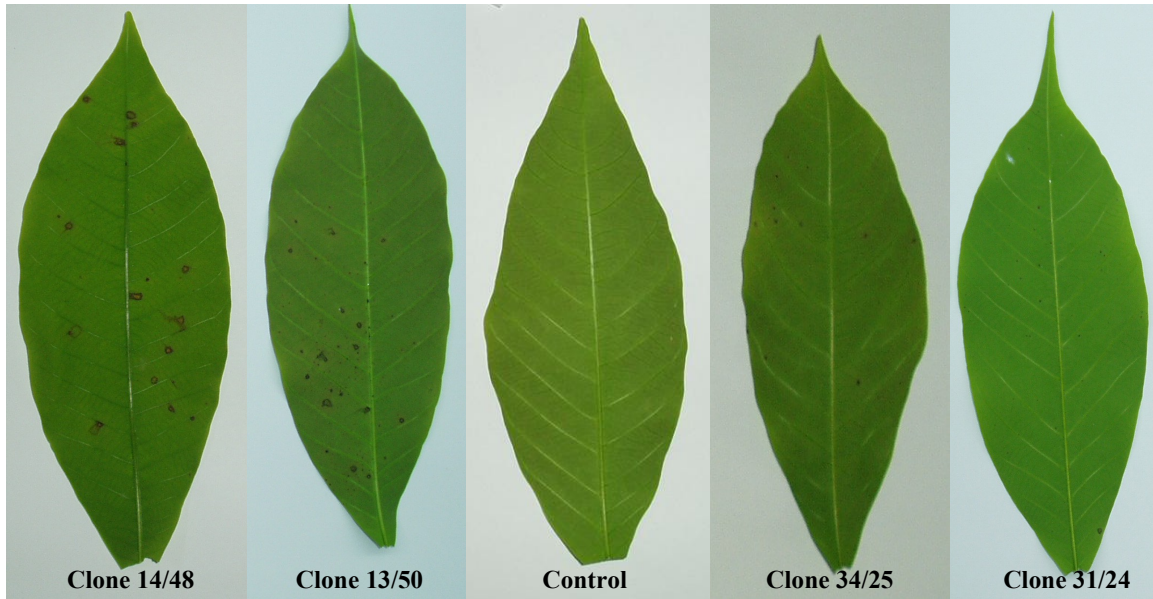
RNA blots were prepared according to standard protocols (Sambrook *et. al*, 1989). Radiolabeled probes were generated by using random primer reaction (Megaprime DNA Labeling System, Amersham). The blots were then exposed to X-ray film for 7 days before developing the film with KODAK developer and fixer.

## **RESULTS AND DISCUSSION**

The differences of severity due to infection of *C. cassiicola* on least susceptible and susceptible clones were obvious. The tolerant clones 34/25 and 31/24 (Fig 4) developed a pin prick lesion that are less in number and far apart after 72 h after inoculation with the spores. Whilst the susceptible clones 14/48 and 13/50 developed a lot more larger lesions indicating the development of the disease.

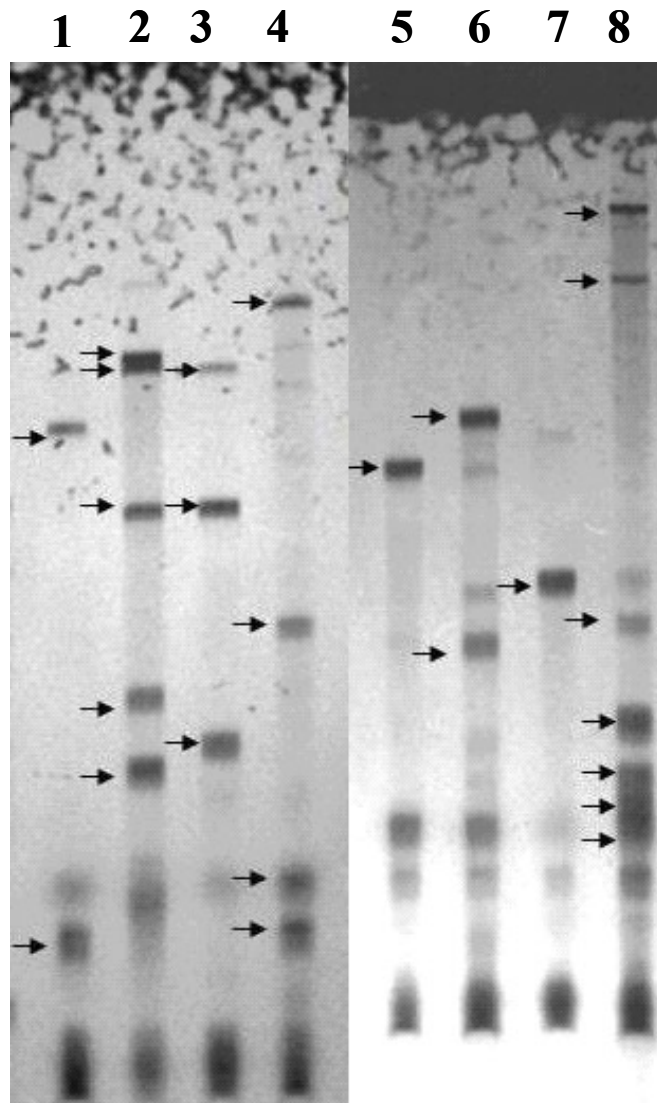
It is difficult to classify any *Hevea* clone as resistant because all clones have a degree of susceptibility to the fungus. The invasion on the susceptible clones appear to be more rapid as compare to the resistant clone and was followed by complete collapse of the epidermis cells. The fungus secretes a cystein rich glycoprotein toxin, cassicolin that was found to be responsible to pathogenicity (Breton *et. al*, 2000). A clone can be resistant to the fungus i.e. invasion of the hyphae is minimal, yet is sensitive to the toxin and vice versa.

An increasing number of cysteine-rich antifungal and antimicrobial peptides have been isolated from plants and in particular from plant seed. These peptides may have an important role to play in the protection of plants from microbial infection, and they could prove to be useful tools for the genetic engineering of fungal resistance in transgenic plants and may be immunized against unrelated pathogens via systemic acquired resistance (Tailor *et. al*, 1997; Durrant and Dong, 2004)



**Figure 4.** Pictures show the level of necrotic lesions on susceptible *Hevea* clones (clones 14/48 and 13/50) and least susceptible clones (clones 34/25 and 31/24) 72 h after being inoculated with spores from isolate CLN 16. The susceptible clones developed larger lesions while the least resistant clones developed smaller lesions that are fewer in number.

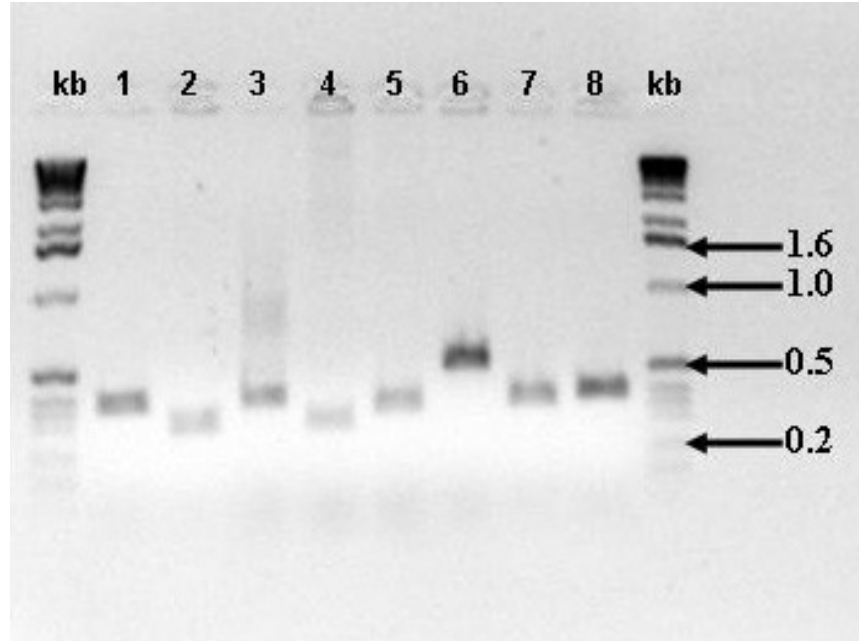
The DDRT-PCR reactions were labeled with radioisotope labels (Fig 5). Several cDNA bands were present in the leaves collected at 0 h as well as leaves after 72 h were excised from the dried gel and re-amplified using the RNAimage™ Kit (GenHunter Corporation) (Fig 6).



**Figure 5.** Autoradiography of a differential display gel. Total RNA was reverse transcribed with anchored (H-T<sub>11</sub>A) and arbitrary primer (H-AP20) that were labeled with [ $\alpha$ -P33] dATP (3000 Ci/mmol).

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|--|--|
| <p>A) Least susceptible clones 34/25 and 31/25: 14 bands</p> <p>Lane 1: Clone 34/25 at 0 h</p> <p>Lane 2: Clone 34/25 at 72 h</p> <p>Lane 3: Clone 31/24 at 0 h</p> <p>Lane 4: Clone 31/24 at 72 h</p> | <p>B) Susceptible clones 14/48 and 13/50: 10 bands</p> <p>Lane 5: Clone 14/48 at 0 h</p> <p>Lane 6: Clone 14/48 at 72 h</p> <p>Lane 7: Clone 13/50 at 0 h</p> <p>Lane 8: Clone 13/50 at 72 h</p> |
|--|--|

The analysis of the differential display patterns obtained from 30 primer combinations had produced 14 specific cDNA fragments in clones 34/25 and 31/24 and 10 cDNA fragments in clones 14/48 and 13/50 (Fig 5)



**Figure 6.** The cDNA inserts were re-amplified via PCR using T7/SP6 primers to confirm successful ligation and transformation processes.  
 Lane 1: cDNA fragment from 27/79 72hr C11-a  
 Lane 2: cDNA fragment from 27/79 72hr G10-b  
 Lane 3-5: cDNA fragment from 27/79 0hr G10-c  
 Lane 6-8: cDNA fragment from 27/79 0hr G10-d

Twenty eight putative clones were sent to First Base Sdn. Bhd for sequencing using T7 and SP6 universal primers. Homology search was conducted using the tblastx program at the NCBI BLAST database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The sequences ranged between 014kb to 036kb and only one sequence had a 57% homology with *Populus* EST from severe drought-stressed leaves (Fig. 7).

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AAGCTTCCAC GTACTATAGG TAATCTTCAG AACTACAAACTTTGGAGGT 50
AGAATGTCCAAATCTACACC AACTGCCTAT AGAAATTCTG AATATTACAC 100
AATTGAGACACCTTCTGTTG AACCAATTACATTTCTTATAG TAGAACTAGA 150
GCTCCAAGAGGAATTGGAAC TTTGGTAAAT CTTCATACAA AAAAAAAAAAAG200
CTTAAGATAT AGACAAAAACGAAGGATATTGTNAGGCGGGGGGTTTCTCT 250
CAC                                                                 253

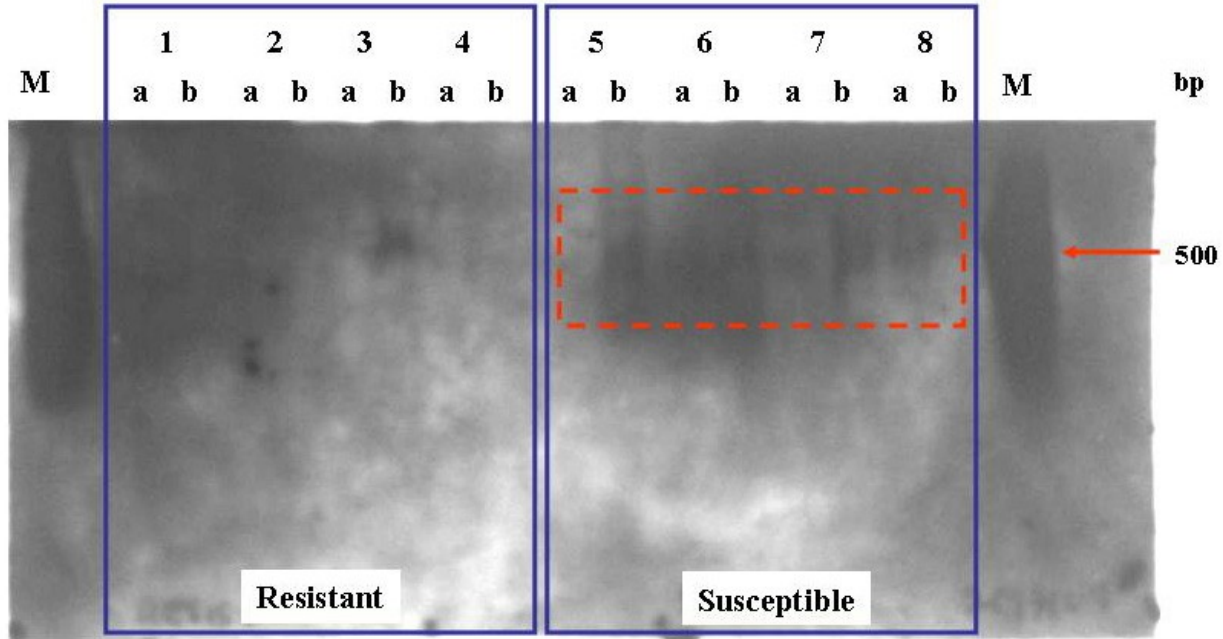
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**Figure 7.** The nucleotide sequence of clone C1-4. The total length of the sequence is 253 bp.

cDNA clones 6-4, 1-4 and 28-3 were used as probes and labeled with chemiluminescent label and hybridized against the prepared Northern blots. Detection via chemiluminescent was also

done using NEBlot® Phototope® and Phototope® Star Detection kit (New England BioLabs, USA).

cDNA probe 1-4 positively highlighted RNA samples from clones that were susceptible to isolate CLN 16 (Fig 8). It appears that the probe was able to hybridize with samples collected both at 0 and 72 hr after inoculation.



**Figure 8.** Northern analysis of *Hevea* clones resistant and susceptible to *C. cassiicola* isolate CLN 16. Total RNA (20 µg/lane) was fractionated on a 1.2% formaldehyde gel and blotted onto nylon membrane for hybridization. a and b represents samples collected at 0hr and 72hr after infection with the fungal spores.

**Lane 1:** Clone KT 17/35  
**Lane 2:** Clone KT 29/76  
**Lane 3:** Clone KT 34/25  
**Lane 4:** Clone KT 34/65

**Lane 5:** Clone KT 13/50  
**Lane 6:** Clone KT 14/48  
**Lane 7:** Clone KT 14/51  
**Lane 8:** Clone KT 27/79

## CONCLUSION

The DDRT-PCR technique was able to detect the differently expressed genes in the susceptible and tolerant *Hevea* plants after they were challenged with the fungus. One of the many cDNA extracted from the experiment was found to have a 57% homology with drought- stressed leaves of *Populus*. This cDNA was observed to hybridize with total RNA from susceptible *Hevea* plants.

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