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Induction of division and differentiation of somatic embryos in the leaf epidermis of *Gaillardia picta*

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Abstract

Somatic embroys and subsequent plant regeneration were obtained from isolated leaf epidermis of Gaillardia picta. Abaxial and adaxial epidermal peels (monolayer) from 45 days old aseptic seedlings were isolated and segments measuring 5 mm x 3 mm were cultured on B_5 basal medium supplemented with various growth regulators such as naphthaleneacetic acid or indolebutyric acid and benzylaminopurine or kinetin. Within 12 h of culture the epidermal cells showed receding of cytoplasm from the walls. After 48 h of incubation 3 or 4 localized zones, each consisting of 3-8 cells that accumulated cytoplasm and stained densely, were observed. Mitotic divisions occurred in these zones on day 3 of culture and localized masses of callus were observed in 95% of the cultures after 10 days. In another 5 days, the callus differentiated somatic embryos or roots, depending on the growth regulators and their concentration in the medium.

Key Words: Epidermis, monolayer culture, morphogenesis, somatic embryos.

Abbreviations used: BAP–6-benzylamiopurine, IBA-indolebutyric acid, Kn- kinetin,NAA–α- naphthaleneacetic acid.

Introduction

De novo differentiation of embryos, organs and organ systems from cultured plant tissues has fundamental implications and numerous applications. Tran Thanh Van (1973a) demonstrated that in Nicotiana tabacum it is possible to induce the formation of roots, shoot buds and flowers either directly or via callus, using diverse explants, ranging from intact organs to thin layers. The latter comprised epidermis and 2-6 subjacent layers of cells. According to her, thin layer systems have several advantages: (1) the explants are small; (2) they have less chances of being influenced by endogenous hormones;and (3) they are comparatively free from inter-tissue correlative effects. Chlyah (1974) employed 1 cm long internodal segments of Torenia fournieri to study the cytological events occurring in the epidermal cells leading to the formation of organs.

In the present communication we report the formation of somatic embroys and plantlets from callus originating from the cultured leaf epidermis (strictly monolayer) of Gaillardia picta.

Material and Method

After testing a large number of plants for the ease with which large epidermal peels can be obtained from both adaxial and abaxial surfaces, Gaillardia picta was chosen as the most suitable system for the present study. G. picta is an annual summer ornamental plant of the tribe Heliantheae (Stuessy, 1977), family Asteraceae. It bears yellow, maroon and mottled capitula. Seedlings were raised in vitro on B_5 basal medium (Gamborg et al., 1968), with 2% sucrose and 0.6% agar under continuous illumination (2000-2500 lux) at 30 ± 1 C. Seeds germinated 8 days after planting. By day 45, the seedlings were 28 cm long and had 15-20 leaves, but no axillary branches.

The first three fully opened leaves (from top downwards), each measuring ca. 40 mm in length x 8 mm in breadth, were excised in a laminar flow chamber and floated on sterile

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distilled water for 5-10 min. Epidermis was peeled aseptically from both adaxial and abaxial surfaces. The appearance of the epidermal peel at the time of culture is shown in Fig. 1. Epidermal segments measuring 5 mm x 3 mm were cultured on semi-solid B_5 basal medium with 0.6% agar and 2% sucrose (control) and with the following combinations of growth regulators and maintained under the same conditions as above:

1. B_5 + α-naphthaleneacetic acid (NAA 10^{-6}M) + benzylaminopurine (BAP 10^{-6}M) Treatment - I
2. B_5 + indolebutyric acid(IBA 10^{-6}M) + BAP (10^{-6}M) Treatment - II
3. B_5 + IBA (10^{-5}M) + BAP (10^{-6}M) Treatment - III
4. B_5 + IBA (10^{-5}M) + Kinetin (Kn 10^{-7}M) Treatment -IV

These treatments were chosen on the basis of observations with intact leaf segments. For each treatment 24 cultures were raised from both upper and lower epidermis. In all 12 cultures were sacrificed for periodic cytological observations and the remaining cultures were maintained to note the morphogenetic responses. The experiments were repeated once. The somatic embryos were germinated in B_5 basal medium, and plantlets were transferred to jiffypots (Jiffy Tarquelltopfe, 45 mm x 20 mm) and maintained in a growth chamber for 15 days prior to potting and transfer to the greenhouse.

For cytological study, the cultured epidermal peels were removed at intervals of 12 h for the first 2 days and 6 h thereafter, stained with freshly prepared acetocarmine solution (1%), and examined under an inverted microscope.

Observations

Cytological changes in epidermal cells

The epidermal peels examined 12 h after culture showed that cytoplasm had receded from cell walls (Fig.2). At the end of 48 h incubation, 3 or 4 zones, each consisting of 3-8 cells had become prominent, showing dense staining with acetocarmine (Fig. 3). Each cell of this zone had a large centrally placed nucleus and a few minute starch grains. The stomata remained open. The cells of the uniseriate multicelled hairs took up a deep stain and the basal cell enlarged and became spherical. The first mitotic division in the epidermal cells was observed on the 3rd day (72 h) after culture. The new wall divided the cell into two equal units (Fig. 4). Interestingly, cells in different zones of the epidermal segments divided synchronously with a mitotic peak on day 4. By day 8, groups of newly formed cells appeared in these zones (dividing zone) in 95% of the cultures (Fig. 5).

The significant changes that were observed in guard cells included breakdown of starch grains, gradual loss of reniform shape, thinning of inner walls and prominence of nucleus (Figs. 6,7). No mitoses were observed in these cells.

Morphogenetic responses

The epidermal peels cultured on control medium failed to show either dedifferentiation of the cells or their proliferation. Neverthelss, in response to all the treatments callus masses appeared in the zones of mitotic activity and covered the entire area of the epidermis in 10-12 days (Fig. 8).

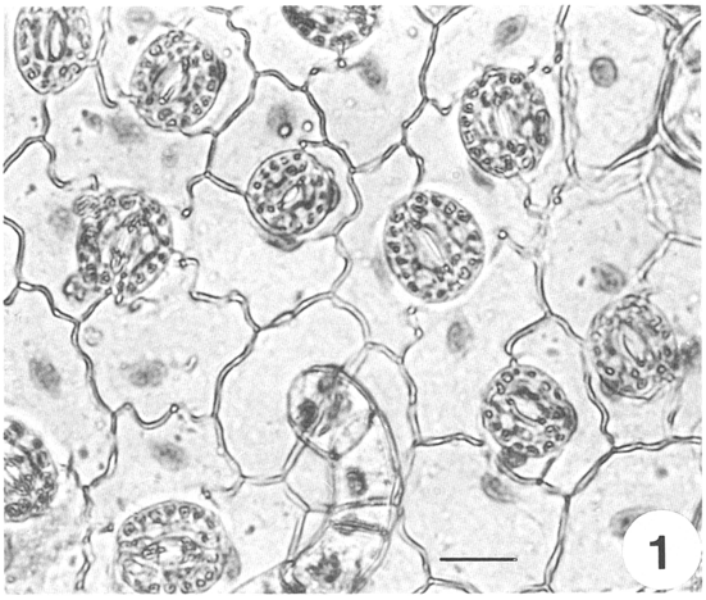
Two distinct patterns of morphogenesis were observed after two weeks. (i) In treatments I-III, somatic embryos differentiated from the callus. Early differentiation of somatic embryos was observed in response to treatment I (Fig. 9), followed by that in treatment II, by the end of the second week in 58% and 50% of the cultures, respectively (Table I). By the end of the fourth week, 83.3% of the cultures showed embryogenesis and 16.7% of the cultures had unorganized callus in treatment I. In treatment II, 33.3% of the cultures showed no organization, 58.4% had only embryos and 8.3% showed both embryos and roots. Callus formation continued for 3 weeks in treatment III and the differentiation of embryos occurred only in the fourth week (Fig. 10, Table I); 66.7% of the cultures showed only embryos and 8.3% both embryos and roots. The remaining cultures had unorganized callus. The embryos were small and spherical to begin with but subsequently became oval to heart-shaped (Fig.11). Development of roots was generally suppressed when BAP was used in the medium (Treatments I-III).

(ii) In treatment IV, callusing was more vigorous as compared to other treatments and tufts of long silvery hairs were noticed on the callus surface by the end of the first week. In the beginning of second week roots emerged from the centre of these tufts (Fig.12) and by the end of the week, 50% of the cultures showed only roots while 8.3% showed both embyors and roots. By the end of the fourth week, 75% of the cultures had both roots and somatic embryos, whereas 25% had only unorganized callus (Table I).

When the somatic embryos were removed and subcultured on to control medium, they developed into plantlets after 25 days. Ten plantlets were transferred to jiffy pots, of which eight plantlets grew further and showed vegetative morphology typical of the source plant (Fig. 13). Further studies on the morphology of the regenerated plants are being carried out.

Discussion

Interaction among cells, tissues and organs has been showed to play an important role in morphogenesis (Tran Thanh Van, 1980; Tran Thanh Van et al., 1974a; Chlyah, 1974). Tran Thanh Van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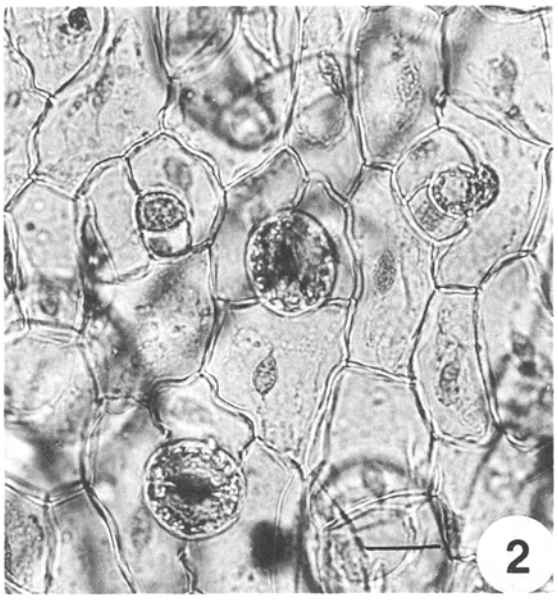
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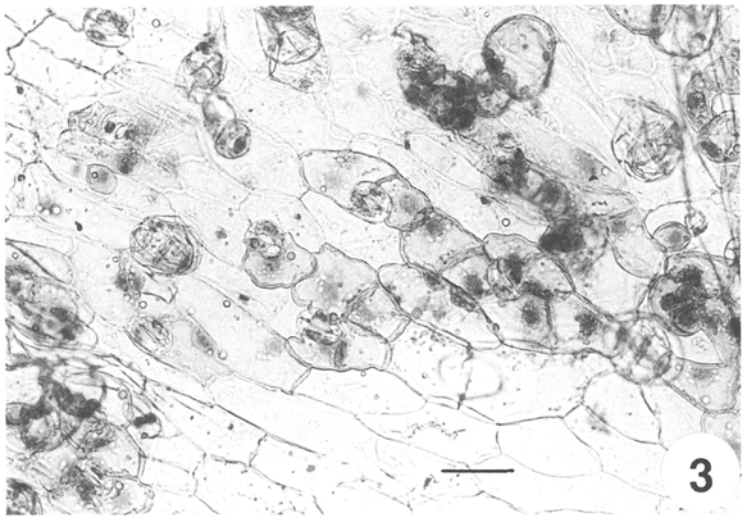
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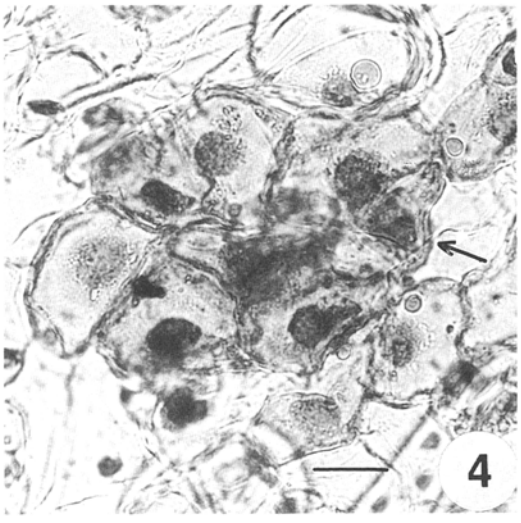
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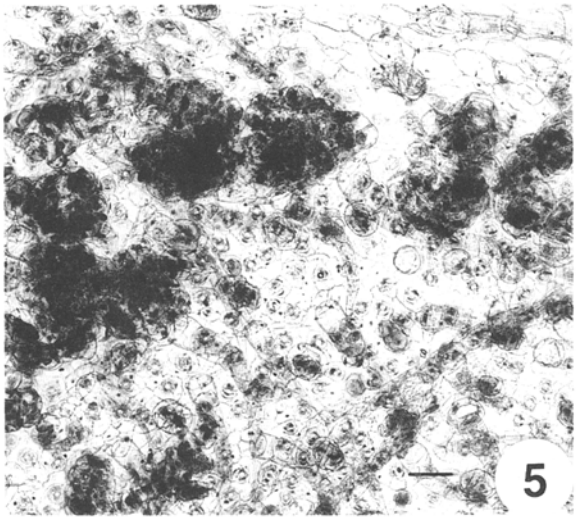
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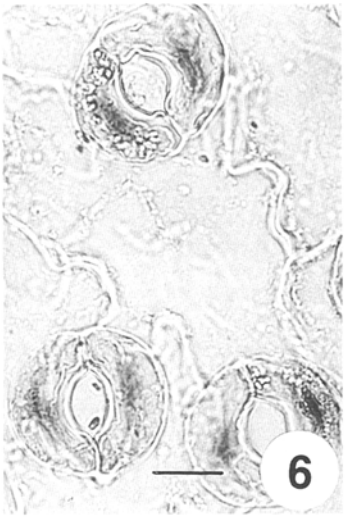
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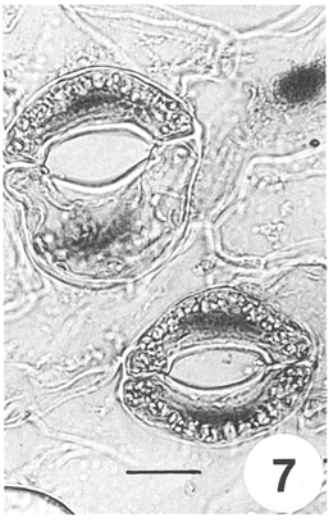
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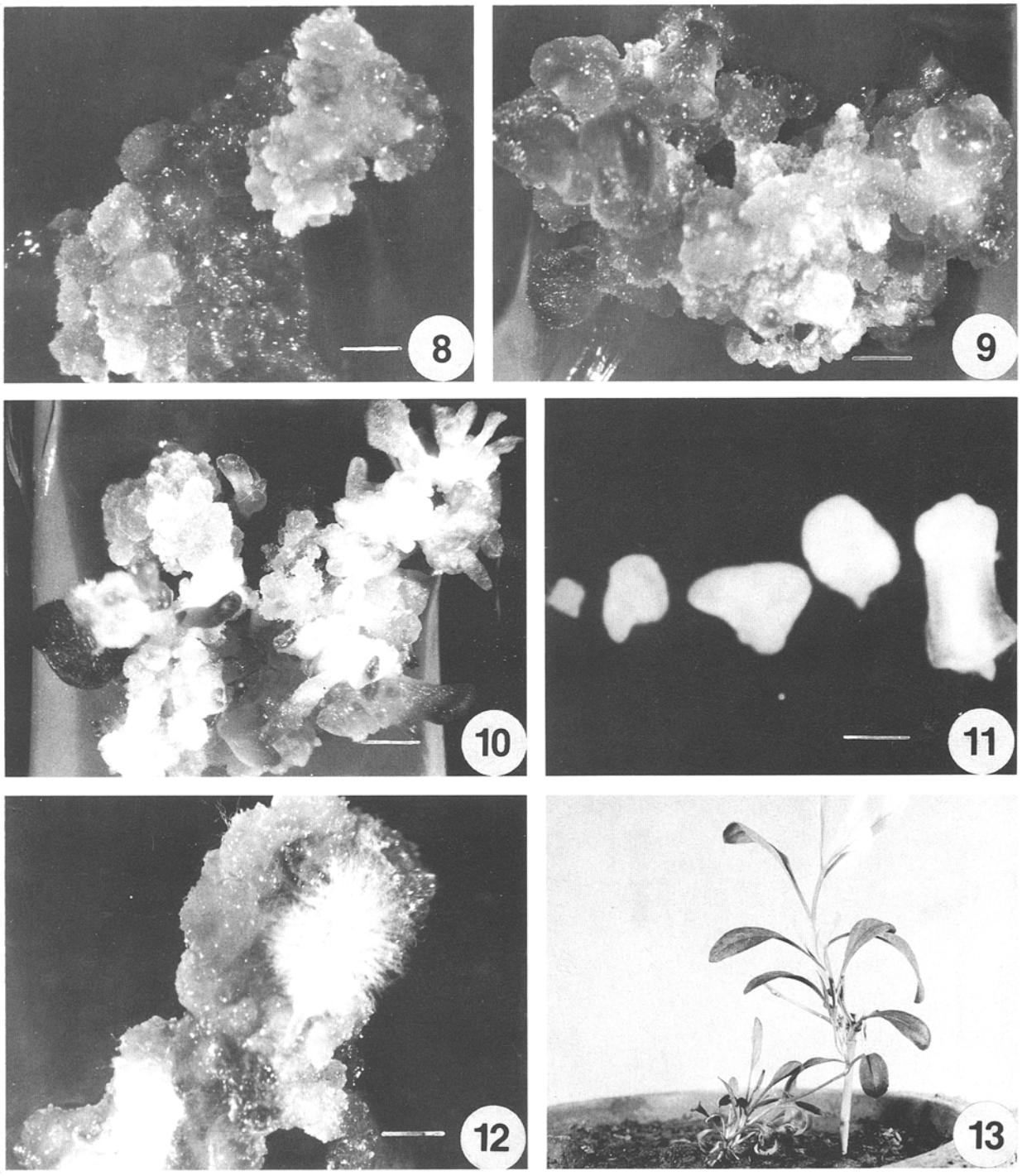

Fig. 1. Epidermal peel (from abaxial surface) at the time of culture (bar = 20μm). **2.** Cultured epidermis showing receding of cytoplasm after 12 h (bar = 22μm). **3.** Differentially stained zones of cells after 48 h of incubation (bar = 20μm). **4.** A zone of mitotic activity. One cell has just divided mitotically (arrow) (bar = 21μm). **5.** Callus masses appearing at different zones of mitotic activity (bar = 86μm). **6,7.** Cytological changes in the guard cells. See text for details (bars = 23 and 21μm). **8.** Callusing epidermal peel after 10 days in culture (bar = 2.6mm). **9,10.** Epidermal peels showing somatic embryogenesis in treatment s I and III respectively. Photographs taken after 2 weeks in treatment I and after 4 weeks in treatment III (bars = 2.1 and 3.3mm). **11.** Somatic embryos isolated at different stages of development (bar = 2.5mm). **12.** Emerging roots from the callus of the epidermal peel in treatment IV. Note the tuft of long silvery hair (bar = 2.5mm). **13.** Regenerated plant 45 days after transfer to the soil.

(1977, 1980) and Tran Thanh Van and Trinh (1978) have demonstrated direct differentiation of floral or vegetative buds, roots or callus from thin layers consisting of epidermis and 2-6 subjacent cell layers in Nicotiana, Begonia, Torenia, and Chichorium, depending on the components of the medium and source of the explant. Chlyah (1974) reported that when only the epidermis was cultured, it merely dried up but when it was placed on epidermectomized stem segments with or without a thin film of agar in between, it was able to produce vegetative buds after the supporting stem segments had formed a callus. In the present study, we have consistently observed that cells in the monolayer of epidermis divide equationally, form meristematic centres and give rise to a callus which may remain unorganized or may differentiate somatic embryos or roots or both. It is important to note that when discs (5 mm in diameter) of leaves of G. picta were cultured on B_5 basal medium with BAP ($5x10^{-6}$M), direct regeneration of plants occurred only from the adaxial surface (data not shown). However, in monolayers the morphogenetic potential of the adaxial and abaxial epidermis is similar. This finding suggests that there is repression of morphogenetic expression of the lower epidermis in an intact leaf.

Receding of cytoplasm from the walls before undergoing division and differentiation lends credence to the view that physiological isolation and insulation is important for differentiating cells (Desikachary and Swamy, 1976). The same phenomenon has been noted by Prema (1988) in the regeneration of somatic embryos from isolated cotyledonary protoplasts of Crotalaria juncea. The occurrence of localized, densely stained zones (dividing zones) which subsequently display the full developmental programme leading to the formation of new plants raises the question whether all differentiated cells are capable of dedifferentiation and morphogenesis or whether this endowment is restricted to certain pre-programmed cells. Chlyah et al (1975) observed that cell division centres do not have a random distribution on the epidermal surface of the stem segments of Torenia fournieri when cultured in vitro. They noted that with respect to the length of the stem segment, the frequency of the cell division centres increases towards the base, which is also the direction of auxin transport. Although such gradients are known to exist in leaves, we found no such polarization of divison centres. The observation that guard cells failed to undergo division emphasizes the view held by physiologists that they are unique, specialized cells (Zeiger, 1983).

Table I. Morphogenesis in cultured epidermal peels : effect of growth regulator combinations

Treatment[a]	Number of weeks after culture: Second week Percentage response[b]				Third week Percentage response				Fourth week Percentage response			
	Unorganized Callus	Callus with only embryos	Callus with only roots	Callus with roots & embryos	Unorganized Callus	Callus with only embryos	Callus with only roots	Callus with roots & embryos	Unorganized Callus	Callus with only embryos	Callus with only roots	Callus with roots & embryos
0 (Control)	0	0	0	0	0	0	0	0	0	0	0	0
I	42.0	58.0	0	0	16.7	83.3	0	0	16. 7	83.3	0	0
II	50.0	50.0	0	0	33.3	58.4	0	8.3	33.3	58.4	0	8.3
III	100.0	0	0	0	100.0	0	0	0	25.0	66.7	0	8.3
IV	41.7	0	50.0	8.3	25.0	0	8.3	66.7	25.0	0	0	75.0

Data based on 24 cultures for each treatment.

a. See text for details of treatments.

b. The cultures were examined under a binocular microscope and scored. The responses were recorded at the end of the week indicated.

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