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Detection and Characterization of Methyl Parathion-resistant *Chlorella protothecoides*

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Methyl parathion, a widely used organophosphorous insecticide inhibits photosynthetic electron transport in chloroplast thylakoids isolated from higher plants (ANBUDURAI et al. 1981) and in Chlorella (SAROJA & BOSE 1983). Furthermore, algal growth is inhibited if the culture medium contains the insecticide (SAROJA & BOSE 1982). During the course of these studies, it was observed that when an autotrophic culture is maintained for several weeks in presence of methyl parathion, the cells develop resistance to the insecticide. This report describes the characteristics of the resistant cells.

MATERIALS AND METHODS

A stock culture of Chlorella protothecoides supplied from the algal culture collection of the University of Indiana (Acc. No. 25), was maintained in a nutrient medium as described by SENGER & OH-HAMA (1976). The cultures were grown in 250 ml culture flask with a total volume of 120 ml medium. Each flask was shaken reciprocally at 120 strokes per minute at 25°C under 3000 lux intensity.

Cell number, Packed Cell Volume (PCV), chlorophyll and carotenoid contents and protein were measured as described earlier (SAROJA & BOSE 1982).

Photosynthesis was monitored in terms of the rate of oxygen evolution polarographically under saturating light intensity using a YSI 4004 Clark type Oxygen electrode (Yellow Spring Instruments Co., Yellow Spring Ohio, USA) connected to an amplifier followed by a strip chart recorder (Toshniwal Bros., India). The assay mixture contained 3.5 ml of the fresh nutrient medium containing 10 µg Chl/ml equivalent of cells. The temperature was maintained at 25°C by circulating water from a thermostated bath around the reaction vessel.

To test the reversibility of resistance, 10 ml of culture containing 8.8×10^6/ml resistant cell were added to two 500 ml culture flasks containing 250 ml of nutrient medium. Methyl parathion was added to a final concentration of 300 µM in one (R-300) of these two flasks and no methyl parathion was added to the other (R-0).

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A third flask inoculated with the same number of sensitive cells served as the control. Cell number (per ml), Chl content (per ml) and photosynthetic O_2 evolution (on chlorophyll basis) were measured on the 7th day and fresh media were inoculated again with 8.8 x 10^6/ml cells. The process was continued for six weeks.

RESULTS AND DISCUSSION

When methyl parathion was added at a final concentration of 300 μm to an exponentially growing autotrophic culture of Chlorella prototheocoides, the cells were completely bleached and the cell number decreased to 60% of the initial cell number (16 x 10^6/ml) in five days (SAROJA & BOSE 1982). When this culture was left without transfer to a fresh nutrient medium, a progressive recovery of the green cells was observed after 30 days. By day 40 the cells (resistant cells) were transferred to fresh nutrient media containing methyl parathion at 300 μM and 400 μM and their growth was observed for the next 20 days (Fig. 1). For comparison a batch of cells were grown in parallel in insecticide free medium (control cells). Fig. 1A shows that while the number of control cells (C-300) decreased in 300 μM culture, the resistant cells in presence of 300 (R-300) and 400 (R-400) μM insecticide grew at a rate which was about 50% of the untreated cells (C-0). The growth of R-400 was essentially the same as that of R-300. Figure 1B shows that while the C-300 cells were completely bleached, the chlorophyll content (per ml) was only slightly inhibited in R-300 and R-400 cells.

These results indicate that the R cells had developed resistance to the insecticide. The Chl a/b ratio in the resistant cells was unchanged as compared to the control cells. The increase in carotenoid content in the resistant cells followed a similar pattern to that of the chlorophyll content (data not shown).

Table 1 shows various characteristics of the resistant cells as compared to the control. The cell number decreased by about 60% and cell diameter increased by 50%. Chlorophyll content and protein content decreased each by 25% and 27% on the basis of culture volume, but increased by 99% and 70% respectively on the basis of cell number. Protein to chlorophyll ratio decreased only slightly (data not shown). Chlorophyll a/b ratio remained unchanged. Dark respiration rate (on Chl basis) was unchanged (data not shown) and photosynthetic rate of O_2 evolution (on Chl basis) was reduced by 50%.

To test whether the observed adaptation was genetic, the resistant cells were transferred to insecticide-free medium and grown for the next 6 weeks or for six successive cultures. In parallel experiments, resistant cells were grown in the presence of 300 μM insecticide and control cells were grown in absence of the insecticide. At zero time the cell number was 8.8 x 10^6 per ml for the three cultures. Fig. 2A shows that the cell number of the resistant cells in presence of the insecticide (R-300 culture) decreased to about 45% of the control in one week as expected and remained at this level. Resistant cells grow in insecticide free medium

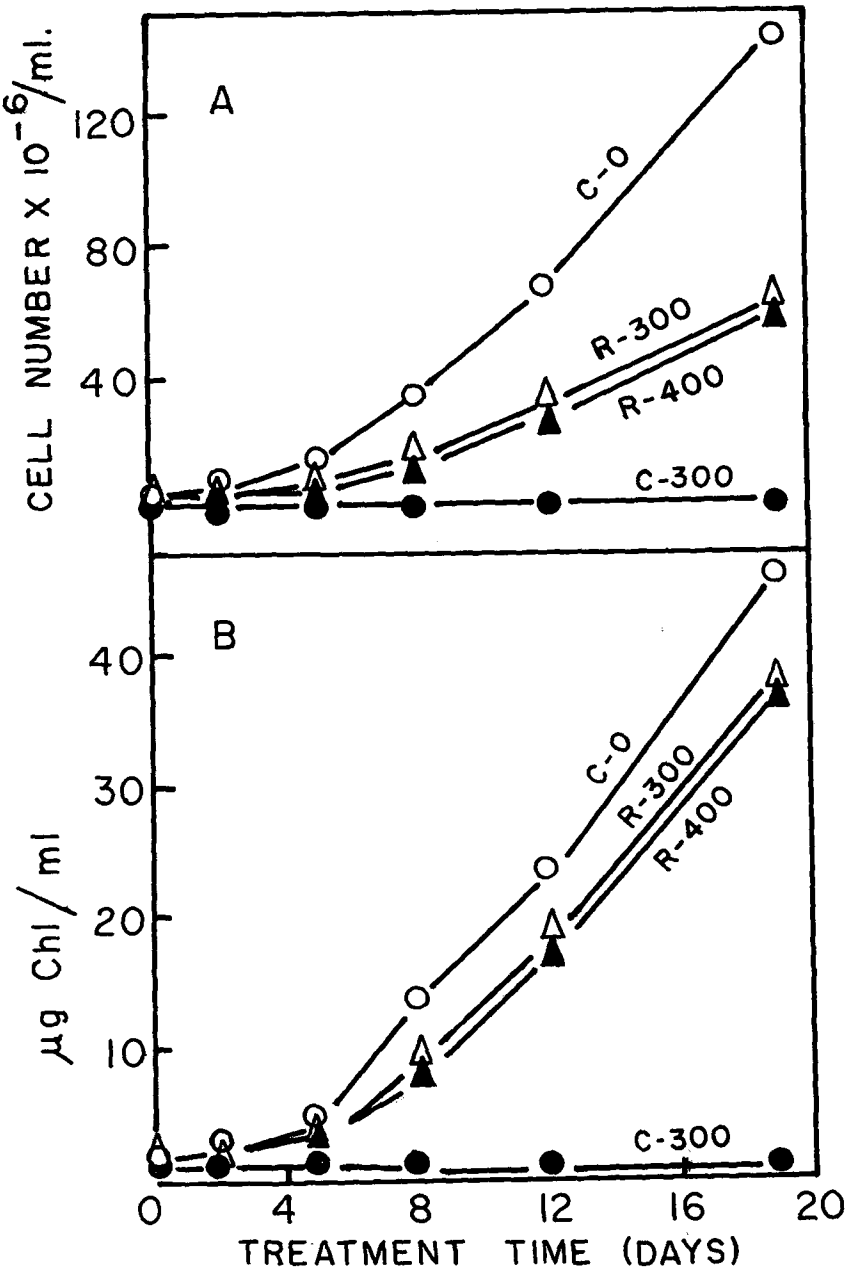

Figure 1. Effect of methyl parathion on cell number (1A) and Chl content (1B) of the sensitive (control) and resistant cultures of Chlorella protothecoides growing autotrophically. C-0, C-300: control without and with 300 µM methyl parathion. R-300, R-400: resistant cells with 300 µM and 400 µM methyl parathion.

(R-0 culture) showed a similar trend during the first week, but started growing faster thereafter and by the 4th week the cell numbers were identical to the control culture. An almost similar pattern of revival was noted in the case of chlorophyll content (per culture volume) as shown in Fig. 2B. It should be noted that on zero day the chlorophyll content in the resistant cell was more than 100% of the control, because the cell density was equal in these cultures and each resistant cell contained more chlorophyll than each control cell (Table 1). The rate of O_2 evolution also revived during the first and the fourth week to the control value and remained the same thereafter (Fig. 2C). These results showed that adaptation was not permanent, suggesting that the mechanism of adaptation is not genetic.

The revived cells exhibited similar growth kinetics as the control in terms of cell number and chlorophyll content (Fig. 3A and 3B). The results also show that the sensitivity of the revived cells to 300 μM insecticide was practically identical to that of the control cells. Figure 3C shows that the rate of photosynthesis during growth and its sensitivity to the insecticide in the revived cells were similar to that in the control cells.

The results described in this report have shown clearly that when Chlorella protothecoides is grown autotrophically in presence of methyl parathion for several weeks, the cells become adapted to the insecticide. The mechanism of adaptation reported herein is not clear at this moment. However, the results on reversibility of adaptation upon growing the resistant cells in insecticide free medium indicate that the genetic make-up of the cells were not changed. Increased PCV, cell diameter, Chl and protein contents per cell indicate inhibition of cell division. This indication is supported by comparatively lower growth rate (in terms of cell number) of the resistant cells. Our preliminary observations (not reported here) suggest that the permeability of cell membrane of the resistant cells decrease drastically to the insecticide as well as to other electron transport inhibitors like DCMU and to many other metabolites. Thus the mechanism of adaptation appears to include a change in the cell membrane permeability in a rather non-specific manner. ROST et al. (1977) suggested a change in membrane permeability for inhibition of cell division and doubling of ploidy in 10^{-6}M ioxynil treated root tip meristem of Pisum sativum. Such a possibility, if present, could explain the results obtained here. It should be noted that there are a few reports on adaptation of green and blue-green algae to herbicides (CALVAYRAC et al. 1979) and insecticides (VAISHAMPAYAN & PRASAD 1981) after organoheterotrophic growth. Reports on adaptation of algae in inorganic media are not found. To our knowledge this is the first report of adaptation of an alga growing in an inorganic medium.

Long term treatment of Chlorella with methyl parathion leads to inhibition of growth and metabolism followed by progressive recovery and final adaptation. It appears that inhibition of cell division and reduced rate of photosynthetic O_2 evolution are the two

Table 1.

Characteristics of the resistant cells as compared to the control.

	Cell No. ml x 10^{-6}	Cell dia. micron	PCV (ul) 10^6 cells	Chlorophyll µg/ml	Chlorophyll µg/10^6 cells	Chl a/b ratio	Protein µg/ml	Protein µg/10^6 cells	O_2 umole mg. chl. h
C-O Control	33.9 ± 3.05	5.00 ±0.15	0.19 ±1.50	13.07 ± 1.89	0.36 ±0.08	2.6 ±0.13	5.5 ±0.04	0.16 ±0.05	204.0 ± 0.45
R-300 Resistant	14.60 ± 4.28	7.68 ±0.14	0.39 ±0.45	9.74 ± 0.50	0.71 ±0.10	2.4 ±0.10	4.0 ±0.03	0.27 ±0.04	97.0 ± 9.86
Change compared to control	-57%	+50%	+103%	-25%	+99%	-8%	-27%	+70%	-52%

The resistant cells were grown in medium containing 300 µM methyl parathion. Fresh nutrient media containing 300 µM insecticide were inoculated once in 5 days with 5 x 10^6 per ml of cells from the previous culture. Just before inoculation of the fresh media, various characteristics were measured and compared with the control. The results of five successive measurements are averaged.

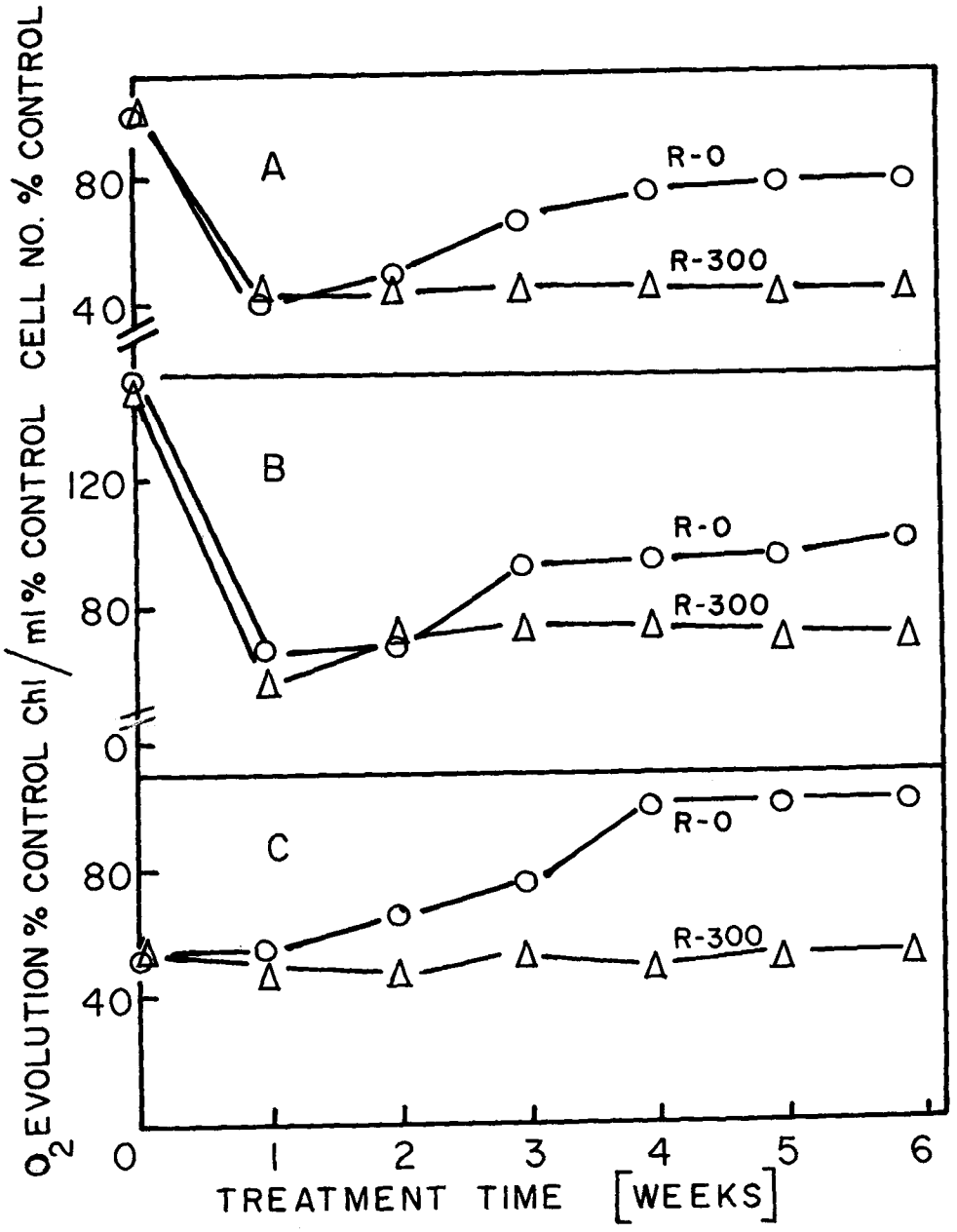

Figure 2. Reversal of acquired resistance during growth of resistant cells in insecticide-free medium with respect to cell number/ml (A), chlorophyll content/ml (B) and the rate of O_2 evolution/mg Chl (C). R-0, R-300: resistant cells without and with 300 μM methyl parathion.

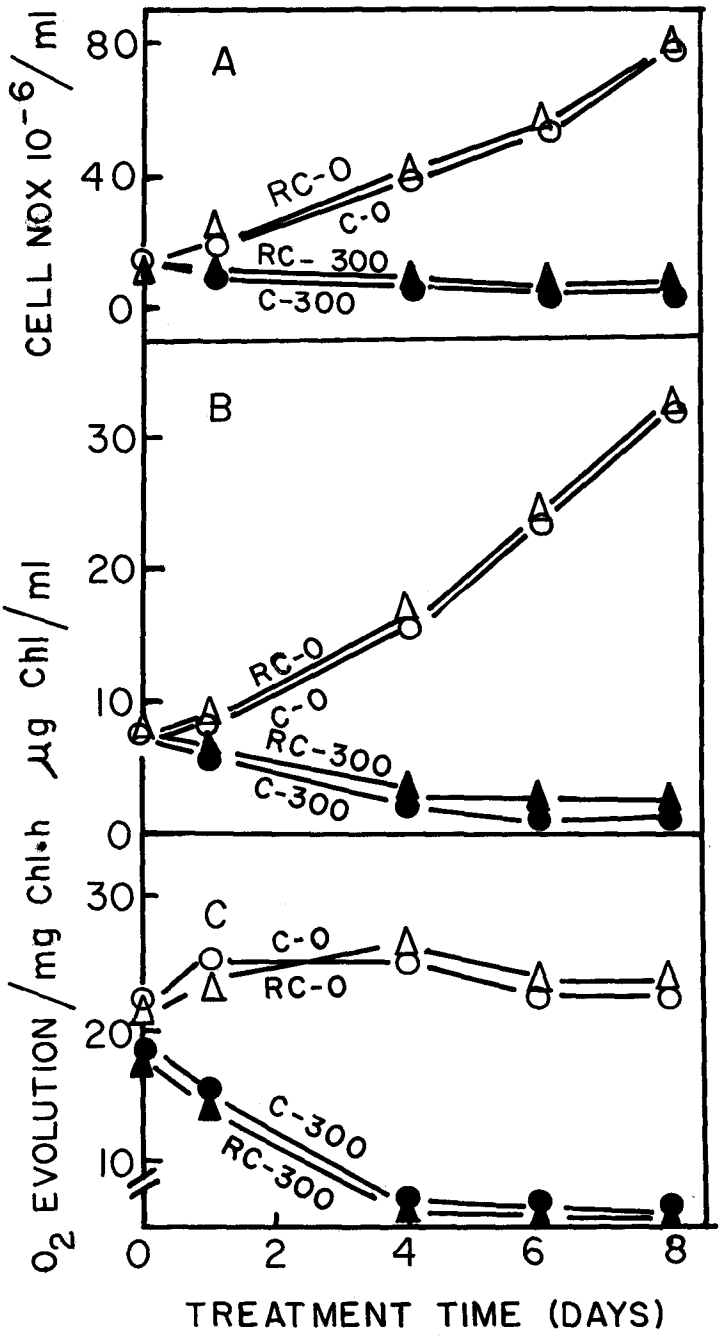

Figure 3. Effect of 300 μM methyl parathion on cell number (A) Chl content (B) and O_2 evolution (C). C-0, C-300: control cells without and with methyl parathion; RC-0, RC-300: Reversed cells without and with methyl parathion.

main characteristics of the adapted cells. Both these effects are possibly related to altered permeability of the plasma membrane. When the adapted cells are resuspended in insecticide-free medium, they revert back to their normal state in four weeks indicating that the acqired resistance is not a genetic change.

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