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# Direct and indirect calorimetric studies of stress responses of chlorella cells to infection with the mycoplasma, *Acholeplasma laidlawii*

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**Abstract**

This paper reports the defence responses of plant cells to the stress of infection by mycoplasma using an algae model of *Chlorella vulgaris* under attack by the *Mollicute*, *Acholeplasma laidlawii*, which is normally a pathogen of animal systems and higher plants. When the two unicellular organisms were mixed, there was a significant rise in the heat flow rate from 30 min after the chlorella cells were mixed with the mycoplasma cells and were thermally equilibrated in the instrument. There were two peaks in the formation of the superoxide anion radical ($O_2^{\bullet -}$). The first one appeared immediately after mixing the two organisms and essentially was completed in 10 min. This coincided with the raised oxygen uptake rate (OUR) for the supply of the superoxide that can destroy the foreign organisms. The second transient "burst" of superoxide anion radical production was observed after 40–50 min, and correlated with both the continued high level of OUR and the observed "heat burst". The increased OUR by the chlorella cells at the early stages of infection may be the trigger for further, more prolonged defence reactions by the cells treated with mycoplasma.

The continued infection with mycoplasma, depressed the rate of growth and division of the chlorella cells. This might be caused by competition of the former for substrates required by the host cells. The heat flow rate was also reduced, reflecting the depressed metabolic activity of the plant cells after prolonged exposure to the pathogen. © 2002 Elsevier Science B.V. All rights reserved.

*Keywords:* Chlorella; Mycoplasma; Heat production; Superoxide anion radical; Oxygen consumption; Defence reaction of algae

## 1. Introduction

In recent years, host pathogen interactions have become one of the foci in phytopathology. As a result, there have been numerous investigations designed to reveal the mechanisms employed in the response of plant cells to attempted infection by many different types of pathogens from the various groups of microorganisms [1–3]. One group that has been less studied in this respect, however, is the class *Mollicutes*, many members of which are commonly known in the pathology of the animal kingdom as well as in higher plants

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as mycoplasma [4]. Even less has been discovered about the putative equivalents in the plant kingdom, sometimes known as the phytoplasma. Therefore, very little is known about the plant defence reactions evoked by pathogenic *Mollicutes*, although many species of them are shown to be positive agents of more than 300 diseases of wide range of plants, e.g. vector-borne witches broom and white leaf [5].

Mycoplasma have a plasma membrane [6,7] but are devoid of a recognizable cell wall. They are the simplest of prokaryotic micro-organisms that are capable of independent reproduction. They have a limited biosynthetic opportunity, only possessing the minimum size of genome capable of self-replication (600–1700 kb) [8]. This causes them to be dependent on the cells of higher organisms for their continued existence. During evolution, mycoplasma have become adapted to co-existence with eukaryotic cells and are facultative parasites of humans, animals and plants [6].

One of the causes of mycoplasma pathogenicity appears to be the competition with the host cells for substrates to use in both energy metabolism and biosynthetic processes. Mycoplasma have become highly dependent on exogenous sources for the majority of their nutrient components and require an effective system to transport them from the host [9]. Despite an apparent restriction in ATP production, mycoplasma not only survive in vivo as extracellular pathogens but appear also to be capable of invading the host cells [6,7] to become intracellular parasites. These cause consequent changes to the host metabolism and successful replication to overcome the host defence systems.

Studies of the molecular and biochemical mechanisms of host pathogen interactions can provide the basis for protection against mycoplasma infection. In this context, the present investigation was aimed at revealing the defence responses of plant cells to mycoplasma infection. In order to have a clear insight to the mechanism, it was thought preferable to use a simple model system of unicellular micro-algal cells like *Chlorella vulgaris* growing in culture at a rate that makes tenable a short experimental period. In the absence of a known pathogenic mycoplasma (sometimes known for the plant kingdom as phytoplasma) for algae, a well-documented animal and higher plants pathogen, *Acholeplasma laidlawii*, was chosen for this study. This permitted an investigation into the metabolic events employed in the defence against the parasite and the subsequent co-existence of them to the disadvantage of the host. In physiological terms, the overall metabolic activity of the organisms was determined by measuring the heat production as the integral variable that reflects the change in all catabolic and anabolic processes.

The alterations in energy metabolism of the unicellular alga, *C. vulgaris*, at different stages of the interaction with *A. laidlawii* and the later penetration of the mycoplasma into the micro-algal cells were studied together with the dynamics of the rate of division of the cells and the heat production during long co-existence of them with mycoplasma. It is unsurprising that the experimental data discussed here indicated the possible correlation of the heat flow rate and the oxygen uptake rate (OUR) by the micro-algal cells in response to mycoplasma attack. The surprise, however, was the evidence that these cells produced the superoxide anion radical as an early response to the infection by the mycoplasma.

## 2. Experimental

### 2.1. Cell culture

The unicellular micro-alga, *C. vulgaris* and the mycoplasma, *A. laidlawii* were the subjects of the investigation. Chlorella organisms were grown in Tamiya medium, pH 6.8–7.2 [10] at 30 °C, and illuminated at $1 \times 10^4$ lx with a light and dark photoperiod of 12 h each. Cell suspensions were bubbled with 0.3% $CO_2$. The optical density was maintained at $1 \times 10^8$ to $1.5 \times 10^8$ cells/ml.

*A. laidlawii* was grown in test tubes at 37 °C with Edward medium, pH 8.0–8.5 [11]. Before each experiment, a suspension of it was diluted in phosphate buffer and centrifuged at $20,000 \times g$ for 10 min at 20 °C. The sediment was resuspended in phosphate buffer, pH 7.2 at a titre of $10^7$/ml viable cells and added to the culture of chlorella in Tamiya medium at 30 °C, in the ratio 1:4.

The numbers of viable mycoplasma cells were determined with the help of both spectrophotometry by measuring the culture density and the isolation of the cells on agar-medium with subsequent counting of

the colonies to express the culture titre in colony forming units (CFU).

As a preliminary control, experiments were made on the influence of a mixed medium of Tamiya with phosphate buffer in ratio 4:1 on the physiological activity of *Chlorella* and *A. laidlawii*. Analysis of the experimental data showed that the heat flow rate of the chlorella suspension in the mixed buffers was identical to that of the control in Tamiya medium alone. The heat flow rate of *A. laidlawii* in the mixed medium was slightly increased in comparison with that of the chlorella culture.

Chlorella cells were infected with mycoplasma at time zero. Mixed samples were quickly divided into three parts and measured for the heat flow rate, the OUR and the quantity of released superoxide in the short term (<2 h).

In the longer term, *Chlorella* and *A. laidlawii* were grown together in 250 ml retorts for up to 21 days to study the influence of chronic mycoplasma infection on the chlorella cells. The suspension was cultured at 30 °C with continuous stirring and illuminated at $3 \times 10^3$ lx.

### 2.2. Analytical measurements

The indirect calorimetric measurement of respiration was made in terms of the OUR by placing 3.2 ml samples in a polarographic apparatus with a Clark-type electrode (SCB of Biological Equipment, Puschino, Russia) at 30 °C measuring OUR for 5 min [12]. Independent samples were taken at every time interval.

The direct calorimetric measurement of heat flow rate of the chlorella cells was estimated using an LKB batch, heat conduction colorimeter (Biological Activity Monitor BAM; successors to LKB are Thermometric AB, Järfälla, Sweden) operated at 30 °C [13]. It was electrically calibrated at the beginning of each day's work. A 1.5 ml cell suspension was placed in a 3 ml glass vial. A control vial contained 1.5 ml distilled water. The thermal equilibration time for the vials was between 15–20 min. So, the onset of measuring the heat production corresponds to 20 min after addition of the mycoplasma to the chlorella.

The quantity of superoxide released by the cells in suspension was detected by incubating them with 1 mM epinephrine for 10 min as described in [14]. Independent samples were taken at each of the stated time intervals. The growth of the chlorella cultures was measured according to the optical density of the cell suspension using a CFC-2 colorimeter (Optical–Mechanical Factory, Zagorsk, Russia) at 670 nm [15]. The data presented in the figures is the average of three experiments each.

## 3. Results

The addition of *A. laidlawii* cells to a *C. vulgaris* suspension was made in order to discover the nature of any possible response by the micro-algal cells in terms of a plant–pathogen interaction. One chosen index of cellular change was direct calorimetry. As can be seen in Fig. 1, the heat flow rate of the chlorella cells treated with mycoplasma was three times greater than in the controls 30 min after mixing them with mycoplasma cells at the time when the contents of the vials had equilibrated with the temperature of the micro-calorimeter. The heat flow rates of the separate control cultures of *C. vulgaris* and *A. laidlawii* in their respective media were close to equality. The initial stages of the burst of heat induced by mycoplasma could not be observed but the phase after thermal equilibration displayed rapid change and was transient with a maximum at 40 min after the addition of the mycoplasma to the chlorella suspension. This peak was followed by a sharp decrease in the heat flow rate at 50–60 min. The observed peak exceeded the heat flow rate of control five-fold and was followed by a reduction of the heat production to the presumed initial level after 60 min.

The data depicted in Fig. 2 exhibit that the early events in the response of the *C. vulgaris* suspension to infection by *A. laidlawii* were very well recorded by the very rapid increase in the production of the superoxide anion radical ($O_2^{\bullet-}$) within the first 5 min, considerably before thermal equilibrium. A second peak of superoxide production was observed 40 min after the initial contact of host and pathogen (Fig. 2.).

The first peak in the formation of the superoxide ion and the rise in the heat flow rate coincident with the second peak in the formation of large amounts of superoxide were accompanied by an increase in OUR of the infected chlorella cells, indicative of the

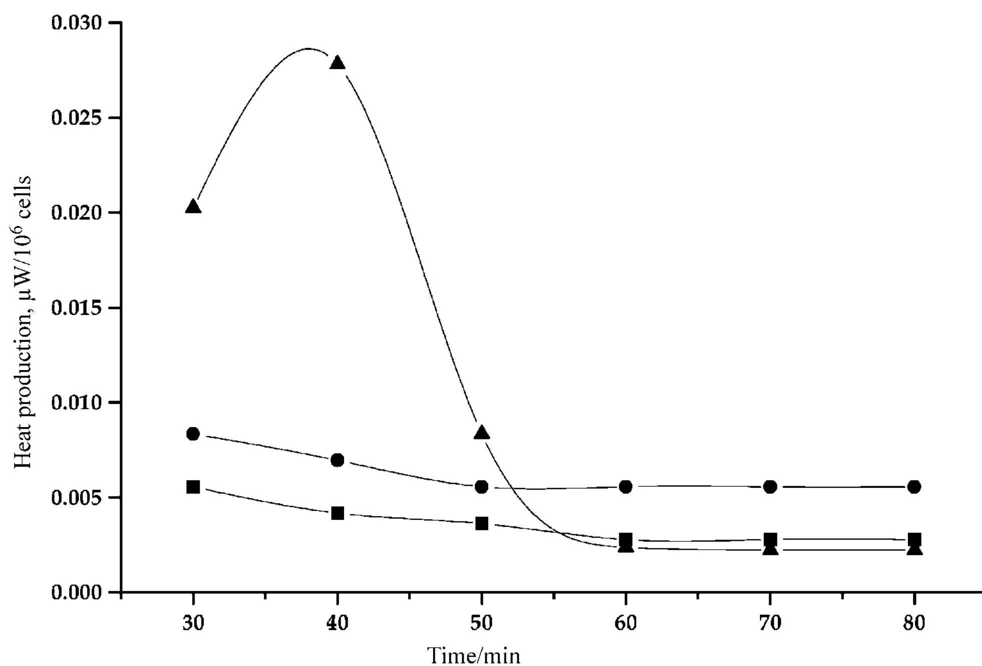


Fig. 1. Change of *Chlorella* heat production rate µW per $10^6$ cells on addition of *Acholeplasma laidlawii*. (■) Control, chlorella in Tamiya medium + phosphate buffer, (●) mycoplasma in Tamiya medium + phosphate buffer, (▲) chlorella + mycoplasma.

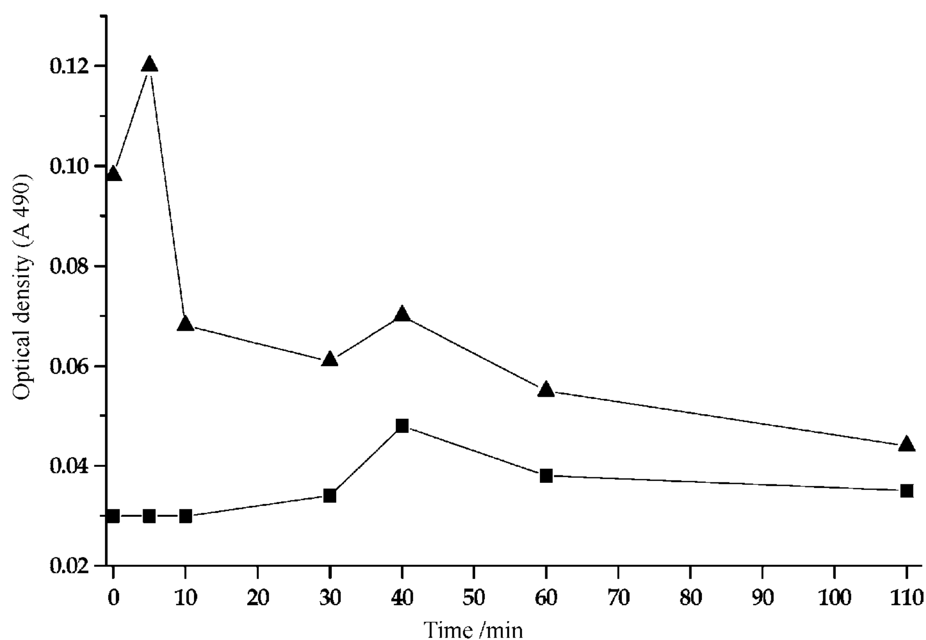


Fig. 2. Superoxide formation in chlorella cells was determined as optical density at 490 nm treated with *Acholeplasma laidlawii*. (■) Control, chlorella in Tamiya medium + phosphate buffer, (▲) *Chlorella* + *Acholeplasma laidlawii*.

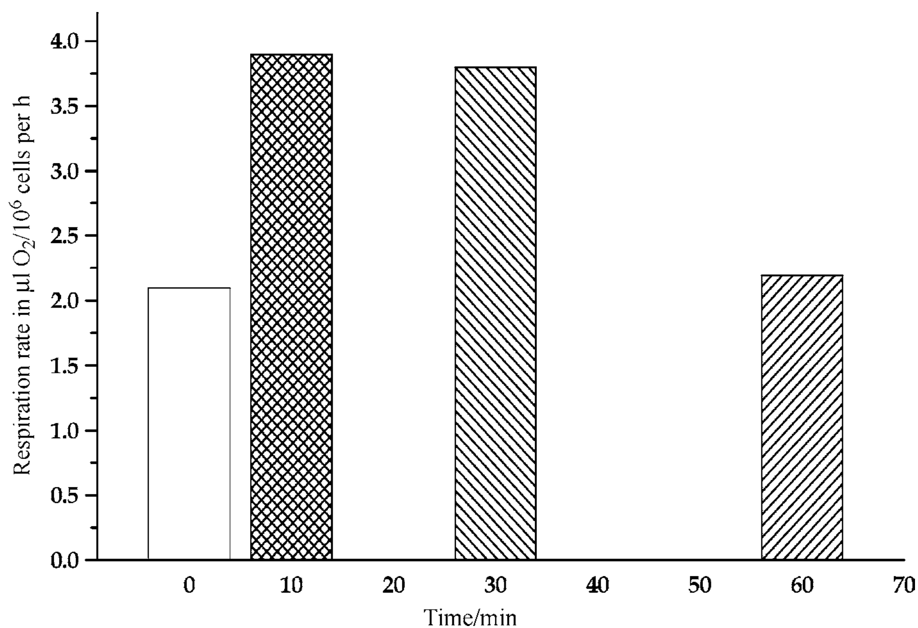


Fig. 3. Respiration rate of chlorella cells ml $O_2\,h^{-1}$ per $10^6$ cells after addition of *Acholeplasma laidlawii* control, □ chlorella in Tamiya medium + phosphate buffer, ▩ 10 min exposition with *A. laidlawii*, ▧ 30 min exposition with *A. laidlawii*, ▨ 60 min exposition with *A. laidlawii*.

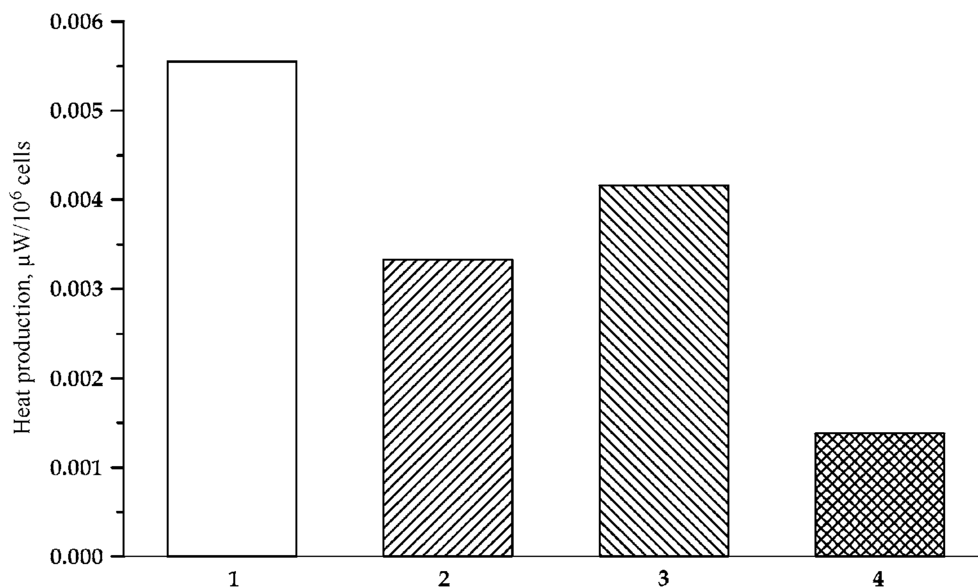


Fig. 4. Heat production rate µW per $10^6$ cells after prolonged co-existence of chlorella cells with *Acholeplasma laidlawii*, □ Control-1, chlorella in Tamiya medium + phosphate buffer (after 5 days), ▨ *Chlorella* + *A.laidlawii* after 5 days exposition, ▧ Control-2, chlorella in Tamiya medium + phosphate buffer (after 10 days), ▩ *Chlorella* + *A.laidlawii* after 10 days exposition.

intensity of respiration (Fig. 3). It can be seen that OUR of the micro-algal cells was double that of the controls within 10 min of introducing the infection. A continued rapid OUR was observed at 30 min but, by 50 min, the rate had dropped to that of the controls.

The pH of the medium gradually decreased, depending on the time of contact of the chlorella cells with the model mycoplasma. At the first moment of contact, pH was 9.15, after 60 min exposure it was 8.65 and at the end, after 120 min, the pH had droped to 7.75.

The next series of experiments was designed to observe the changes in the heat flow rate during the prolonged co-existence of *C. vulgaris* and *A. laidlawii*. It was known that the cell density of the suspension was an important factor in the heat dissipation [21]. Growth and development of chlorella cells were depressed when they were exposed to a prolonged co-existence with mycoplasma (Fig. 5). The heat production of these cells decreased with time compared with that of actively growing cells that had no infection. Indeed, the heat production was decreased by about 40 and 70% from the control at the corresponding 5 and 10 days (Fig. 4).

## 4. Discussion

As a general feature, plants react to an attack by a pathogen with a cascade of stress responses. Various pathogens as well as other stress factors have been shown to cause the so-called "oxidative burst" and, in terms of overall metabolism, the "heat burst" [1,14,22,23]. The oxidative burst is defined as the rapid and transient release of large amounts of active oxygen species (AOS), including the superoxide anion radical, which in plant cells mainly occurs on their surface. It is manifested as a rapid increase in OUR. Although, the burst is well known from work on higher animals and plants, for instance from the response of mammalian phagocytes to a bacterial attack [16], the current findings are new in two respects. First, they establish that the relatively primitive micro-algal cells of *C. vulgaris* possesses a defence mechanism at least similar to that found in higher plants. Secondly, they show that mycoplasma not usually associated with the aquatic mode of life can infect foreign cells of algae. Although, it would seem reasonable that the superoxide anion radical was derived from oxygen metabolism by the micro-algal cells, it is not possible to exclude the chance that the mycoplasma contributed to the total amount of the anion in the mixed cultures. In mycoplasma monoculture, however, there was no detectable superoxide.

The oxidative burst is a part of hypersensitive response of plant cells treated with pathogen. There is a keen discussion in the literature on the sources of AOS during the oxidative burst in plant cells under the pathogen challenge [1,3,24]. At present, it is well established for land plants that the redox system of the plasma membrane and cell wall peroxidase of plant cells make major contributions to total AOS. However, the sources for its formation in algae have not been studied in depth, and therefore are currently under investigation.

It has been reasoned that the initial signals of the awareness of pathogen infection in plants are universal and result from shifts in ion homeostasis during the early stages of the cell stress response [25,26]. It is obvious that the interface between the pathogen and its host is the cell wall and its subjacent plasma membrane that interacts with the cortical cytoplasm that is rich in cytoskeletal elements. It is possible that, in addition, the initial stage in the infection elicits changes to the active transport of ions and facilitated diffusion of glucose in the plasma membrane as well as to the activity of ecto-ATPases. All of these processes are energetic in nature and it is reasonable to conjecture that there is increased activity of them on pathogen attack. This might result in increased respiration but it is not possible to distinguish between oxygen consumed in this purpose and that needed for the immediate production of AOS for the defence of the micro-algal cells. Both would be represented in the oxidative burst. It might be considered, however, that the physiological changes required in order to combat the infection would be a longer term than the transitory production of AOS for the destruction of the source of infection, in this case the mycoplasma. It is reasonable to suppose that, by its very nature, AOS is as harmful to plant cells as it is to the animal tissues, where it causes inflammation [16].

It is very interesting that there were two phases in the production of the superoxide anion in the mixed cultures. The first burst of superoxide formation was very rapid and the anion had dissipated within 10 min

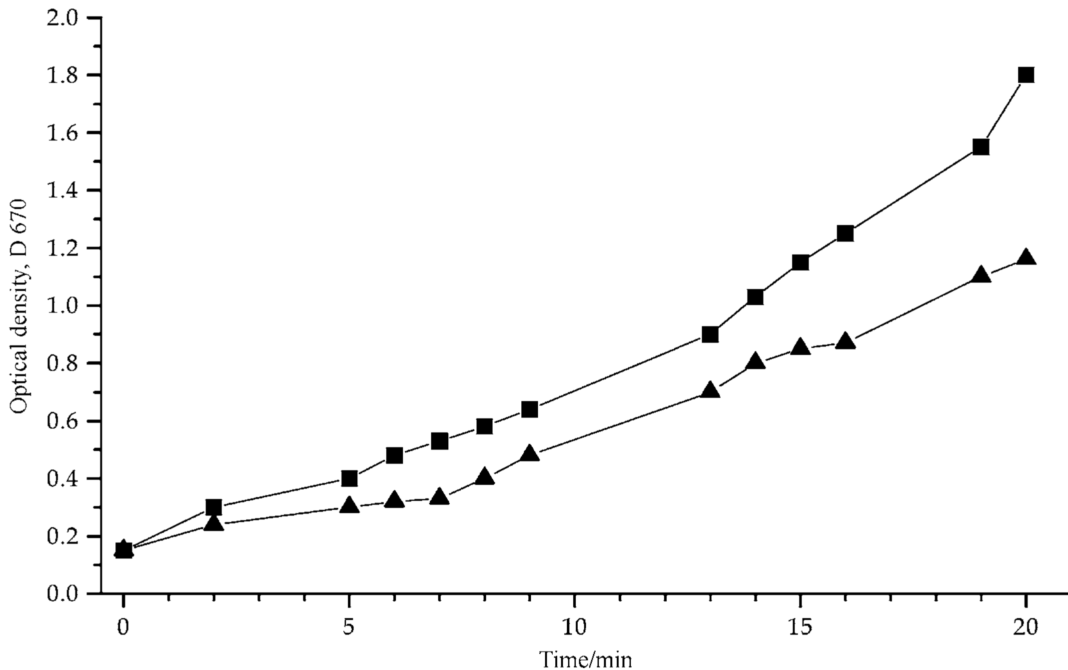


Fig. 5. Effect of *Acholeplasma laidlawii* on growth of chlorella was determined as optical density at 670 nm. (■) Control, chlorella in Tamiya medium + phosphate buffer, (▲) *Chlorella* + *Acholeplasma laidlawii*.

of the first contact between the chlorella and the mycoplasma cells (Fig. 2). This result was manifested in the increased OUR at 10 min (see Fig. 3). The second "peak" is more accurately described as a plateau because the peak associated with it was also seen in the controls. The continued high level of superoxide production at times up to 60 min was naturally associated with raised OUR, necessary for its formation. It seemed to be correlated with a heat burst that was evidenced 30 min after the infection. As seen in the micro-algal cells, the response to the mycoplasma attack by plant-like defence reactions is related to the formation of AOS, in particular superoxide. The increase in OUR and the generation of a large amount of heat are mainly a reflection of this requirement for the generation of $O_2^-$ ions from molecular oxygen in an energetic process that produces heat in the cycling of NADH.

It is thought that chlorella cells attacked by mycoplasma release a significant amount of glycolate that acidifies the medium as part of the nonspecific responses of plant cells to infection. A greatly enlarged release of cellular glycolate has been shown to occur under the action of high temperature and salt stress [17–19]. Also, one cannot exclude the possible formation and subsequent excretion of lactate formed during glycolysis from mycoplasma [20].

A decrease in the activity of energetic and metabolic processes has been observed during the prolonged contact of chlorella cells with mycoplasma. The levels of heat flow rate and of superoxide production decreased to the control level (see Fig. 4), while there was a reduction in the growth (see Fig. 5), and development of the micro-algal cells. Perhaps this depression of growth during the prolonged contact of *Chlorella* with *A. laidlawii* was caused by competition for substrates, especially in the case where some of the mycoplasma cells may have gained access into the unicellular algae. The idea is that the phytopathology upon the mycoplasma infection is related to mechanisms to provide for the prolonged preservation of mycoplasma in the host organism (persistension). In support of this, possibility is the fact that mycoplasma have been shown to exhibit reactive genetic variability that allows them protection from the host immune system [8].

## 5. Conclusions

Based on the above data, it was concluded that the formation of the superoxide anion by the chlorella cells in response to mycoplasma infection is an important part of their defence reactions. Probably, the source of the superoxide is NAD(P)H oxidase for which the cells have to produce electron donors. This process requires cellular energy that is manifested as the increase in the heat flow rate of the chlorella cells that are under stress.

It is possible that, besides the oxygen metabolism as an immediate reaction to stress, some of the raised oxygen consumption may have been due to the increased respiration to meet the energy needs during the stress response for the maintenance of ion homeostasis by active ion transport as well as for the synthesis of proteins, including ecto-ATPases [25].

With the prolonged infection by the mycoplasma (5–20 days), the growth of the chlorella cells is reduced possibly by penetration of mycoplasma into chlorella cells and parasitising of some of metabolites of host cells.

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