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Effects of Zinc and Copper on Male Gametophytes of  
Flowering Plants

Dk Rosinah Pg Hj Damit

Submitted in fulfilment of the requirements for the degree  
of Master of Science

Department of Biological Sciences

University of Durham

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ABSTRACT  
Dk Rosinah Pg Hj Damit

The Effect of Zinc and Copper on Male Gametophytes of Flowering Plants

- 1) The aim of this study was to investigate the effect of heavy metals in the development of male gametophytes in flowering plants. Several experiments were conducted to find the best germination medium for the maximum percent germination and pollen tube growth of pollen. Temperature was also found to be an important factor influencing pollen tube growth.
- 2) Three plant species were used in this study. They were *Kalanchoe fedtschenkoi*, *Brassica rapa* and *Hippeastrum*. The optimum germination medium for the pollen under study was found to be species-specific.
- 3) The best *in vitro* germination medium for the maximum percent germination and pollen tube growth for *Kalanchoe fedtschenkoi* was 10% PEG and 10% sucrose, while for *Brassica rapa* dwarf2 it was 15% PEG and 10% sucrose.
- 4) A 5°C difference of germination temperature was shown to alter pollen tube growth length in two cultivars of *Brassica rapa*.
- 5) Presence of zinc in the germinating medium led to reductions in both percent pollen germination and pollen tube length growth in *Kalanchoe fedtschenkoi* at high concentrations of zinc. In contrast, *Brassica rapa* dwarf2 showed a greater reduction in percent pollen germination than in pollen tube length.
- 6) There was a large inhibitory effect of copper on both the percent pollen germination and pollen tube length of *Brassica rapa* dwarf2.
- 7) Cytoplasmic streaming was found to be a useful indicator of the possible toxicity of heavy metals. The movements of granules on the cytoplasm in the pollen tube slowed down when pollen was incubated in germination medium containing heavy metals. Thus this approach provides a sensitive assay for the effects of heavy metals such as zinc and copper upon male gametophytes.
- 8) *Brassica rapa* dwarf2 grown in water culture medium containing heavy metals showed a reduction in *in vivo* pollen tube growth. Thus, metal uptake by plants from the growing medium does result in metal reaching the male gametophyte and can in turn result in reduction of pollen tube germination and growth, thus potentially markedly affecting reproduction.

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## List of Abbreviations

ANOVA	analysis of variance
Conc.	concentration
FCR	fluorochromatic reaction
Fig.	figure
IAA	indole acetic acid
l	litre
M	molar
mg	milligram
mM	millimolar
NS	non-significant
PAR	photosynthetically active radiation
PEG	polyethlyene glycol
PG	pollen germination
ppm	parts per million
PTL	pollen tube length
Temp.	temperature
UV	ultraviolet
V	violet
w/v	weight per volume
µm	micrometre
%	percent
°C	degrees Celsius

## **DECLARATION**

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other University or institute of learning.

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# Chapter I

## INTRODUCTION

### 1.1 General Effects of Heavy Metals on Plants

Heavy metal toxicity of plants is important because plants act as primary producers in all the food chains. Due to industrial pollution and other man-made activities, phytotoxic amounts of metals like Zn, Cu, Cd and Pb can contaminate the soil. These elements interfere with the most important physiological processes such as respiration and photosynthesis affecting plant development in general. Growth inhibition and reduction of biomass production are general responses of higher plants to metal toxicity (Lepp, 1981).

#### 1.1.1 Heavy Metal Contamination of Soil.

Among the great variety of substances entering the soil as waste products mainly of man-made activities, heavy metals especially create long-term problems. Not only do they accumulate in organisms and thus circulate in food chains, they also remain in the ecosystem in dangerous concentrations for longer periods in sediments. The soil covering ore-bearing rock or near slag heaps contain heavy metals, which include zinc and copper, in amounts toxic to most plants. Heavy metal contamination also occurs in industrial zones, where the sources include refuse dumps and sewage sludge. Another source of zinc and copper is emission of dust from the metal processing industries (Walter, 1995).



### **1.1.2 The Uptake of Heavy Metals by Plants and their Toxic Effects:**

The uptake of metallic elements by plant cells, especially in the roots, is facilitated by appropriate mechanisms for their transport and accumulation, since several heavy metals are in fact required by plants as microelements, such as Mn, Zn, Cu, Fe and Mo. The plant cannot, however, prevent toxic elements from entering by the same mechanisms. The toxicity of heavy-metal ions is due chiefly to their interference with electron transport in respiration and photosynthesis, the inactivation of vital enzymes, as a result of which energy status is lowered, decreased uptake of mineral nutrients, and reductions in growth.

## **1.2 Sources and Availability of Copper to Plants.**

It is widely believed that over 90 % of the available copper pool is in the form of organo-copper complexes (Geering and Hodgson, 1969; Hodgson, Lindsay and Trierweiler, 1966). The relative availability of copper in a soil will determine plant response. Soil copper excess is usually confined to specialised situations. Soils derived from copper-bearing ore bodies may contain very high copper levels, and occur in many parts of the world. These soils may often support a specialised flora; in the Zambian copper belt, the occurrence of *Becium homblei* is characteristic of high copper soils (Drew and Reilly, 1972) and has been used for biogeochemical prospecting.

Copper excess can also arise as a consequence of industrial or agricultural activities. Copper smelting and refining industries, manufacturers of copper products and sewage disposal processes all contribute to all man-made input of copper to the biosphere. Finally, use of contaminated sewage sludge as a soil amendment can be responsible for introduction of excess copper to farm land.

### 1.2.1 Root Uptake and Distribution of Copper with the Plant

Copper uptake by whole plants has been widely studied, from the view point of both deficiency and excess. The main feature of this process is the concentration of copper in the root system. Pettersson (1976), using cucumbers (*Cucumis sativus*), showed that, from a range of metals tested, copper had the second lowest shoot/root metal ratio after 12 days growth in metal containing nutrient solution. Similar observation has been made for tomato (*Lycopersicon esculentum*) (Whitby and Hutchinson, 1974). In the roots, staining techniques have indicated that copper may be accumulated in epidermal cells, and also in the endodermis and pericycle (Brams and Fiskell, 1971). Copper-tolerant plants of the grass *Agrostis tenuis*, showed accumulation of copper in the cell wall. However, this type of investigation has little to tell about the situation in the intact root, as the disturbances of the cells and cell contents during fractionation could result in a redistribution of copper, which, with its high affinity for organic binding sites, may concentrate in tissue fractions offering the highest proportion of such sites (i.e. cell walls) and competitively displace other ions which may have previously occupied these sites (Lepp, 1981).

Macnair (1981), who compared tolerant and non-tolerant lines of *Mimulus guttatus*, found that tolerant lines take up more copper into the roots and shoots than non-tolerant lines, but the effect of variation in degree of copper tolerance on copper uptake is unknown. The variation in degree of tolerance is thought to be controlled by hypostatic modifiers (Macnair, Smith and Cumbes, 1993; Smith and Macnair, 1998). In further work (Tilstone and Macnair, 1997) showed that tolerance genes cause the plants to accumulate more copper in their roots, while the modifier genes cause a change in the partitioning of copper between roots and shoots, with the most tolerant having a lower root concentration, but higher shoot concentration. The tolerant line plus increased modifier gene accumulates copper to a significantly higher concentration in its shoots than the other lines, whilst the least tolerant line (with decreased modifier gene) has a higher root copper concentration than the other lines. However, the tolerant line plus increased modifier gene accumulates a high percentage of symplastic root copper. They

concluded that the tolerant line plus modifier gene translocates more copper since translocation requires movement of copper across the endodermis via the symplast.

Tilstone and Macnair(1997) categorise the patterns of metal uptake at the whole plant level into three: true exclusion, shoot exclusion and accumulation. The pattern of metal uptake by accumulation is recently of considerable interest. Accumulators that concentrate exceptionally high levels of metals in the shoots have been categorised as hyperaccumulators and have shoot dry matter contents of 0.1 % metal in the case of copper (Baker and Brooks, 1989). In recent years, hyperaccumulation has attracted considerable interest because of the potential to use plants to remove metals from contaminated soils *in situ* (phytoremediation). Ebbs *et al.* (1997) conducted a study on the use of terrestrial plants that absorb heavy metals from the soil and concentrate them in the easily harvestable shoot tissues as an alternative remediation technology. They screened a number of different plant species and cultivars for their ability to grow on nutrient solutions containing high levels of Zn, Cd and Cu which have similar mineral content to that of contaminated soil, as well as for their ability to accumulate the metals in the shoots. They found that the best plant species for phytoremediation were plants that are fairly tolerant to toxic soil conditions and produced reasonable shoot biomass, and also exhibited the ability to accumulate moderate amounts of these metals in the shoots. These studies on tolerance of pollen have shown that some plants are known to have become adapted to growing on soils with high heavy metal concentrations.

### **1.2.2. Role of Copper in Plant Metabolism**

Copper was first demonstrated to be the essential element for plant growth by Sommer (1931) and by Lipman and MacKinney (1931). Since then copper has been shown to act as an important factor in several biochemical processes. Many enzymes have been shown to require copper as a co-factor (Evans and Sorger, 1966) and copper also has a strong affinity for other non-catalytic proteins. In plants, enzyme systems known to require copper are chiefly oxidases.

In photosynthesis, copper is an essential constituent of plastocyanin, a copper protein isolated from higher plants and green algae (Bishop, 1964). This molecule accounts for 50 % of the total chloroplast copper content, and the two copper atoms are an integral part of the molecule's structure, being unaffected by adding chelating agents. Plastocyanin is an intermediary in the electron transfer process linking photo-systems II and I (Bishop, 1966). Copper supply has been shown to interfere with floral initiation in various *Lemna* species (Hillman, 1964). There is some relationship between copper, IAA-oxidase activity, high IAA levels and long days for floral initiation in *Chrysanthemum morifolium* (Tompsett and Schwabe, 1974). There is also an observation on copper inhibition of auxin transport in roots (Mitchell and Davies, 1975).

### **1.2.3. Symptoms of Copper Excess in Plants**

Although it is clear that copper forms an essential function in the metabolic activities of higher plants, when in excess (more than optimum) it becomes toxic to the plants. Concentrations higher than 1  $\mu\text{M}$  in the plant are increasingly toxic to plant tissues (Gross, Pugno and Dugger 1970). Higher plants develop necrosis in the presence of relatively low concentrations of copper (Repp, 1963).

Copper toxicity is generally manifested as a general chlorosis and stunting of growth (Foy, Chaney and White, 1978). The chlorotic condition may result from iron deficiency, as foliar application of inorganic  $\text{FeSO}_4$  (Hewitt, 1953) can alleviate this condition. Analysis of foliage from such chlorotic plants reveals a low iron content (Smith, Reuther and Specht, 1950; Roth, Wallihan and Sharpless, 1971). Taylor, Ashmore and Bell (undated) have also laid down the most common symptoms caused by copper which are very similar to iron deficiency. The symptoms are described as young leaves bleached and having interveinal chlorosis (veins remain as fine green lines). Ebbs and Kochian (1994) investigated the individual and combined toxicities of zinc and copper to *Brassica* species that might be used to remediate heavy metal-contaminated

sites. These authors found that copper was more toxic than zinc, and exposure to both heavy metals induced micronutrient (iron) deficiency in the plants, as well as causing a significant inhibition of root growth and a decrease in the accumulation of each metal in the shoots.

Crop stunting due to copper excess can arise from a combination of factors. These include specific effects of copper on the plant, antagonism with other nutrients, or reduced root growth and penetration into the soil (Foy *et al.*, 1978). In the case of copper, toxicity is experienced initially in the root tips (Brams and Fiskell, 1971) with subsequent inhibition of the development of lateral roots. Such a restriction in root length could lead to macronutrient depletion in the restricted rooting zone, and a consequent growth inhibition. Early growth may be normal, but later growth chlorotic (Reilly and Reilly, 1973).

In the recent investigation on the effect of heavy metals on resource allocation in *Potentilla anserina* and its reproductive output, Saikkonen *et al.* (1998) found that heavy metals decreased the total biomass of the plants and number of flowers and ramets produced. They observed that only 50 % of the plants grown with the higher level of heavy metals produced flowers. However, at lower heavy metal treatment, the number of flowers per whole plant was apparently lower in the later season of flowering.

#### **1.2.4 Effects of Excess Copper on Metabolic Processes**

The basic deleterious effect of copper on growth is related to the root system (Mukherji and Gupta, 1972; Hallsworth, *et al.*, 1965). Das Gupta and Mukherji (1977), in a more extensive investigation of copper effects on rice (*Oryza sativus*) seedlings, demonstrated numerous effects which could be attributed to excess copper. These included reduced nucleic acid content, especially in the embryo, reduced activity of both  $\alpha$ -amylase and RNase and reduced protease activity in the endosperm. The most pronounced effects occurred at 5 mM CuSO<sub>4</sub> treatment.

Heavy metals are well known inhibitors of enzymes and copper binds strongly with sulfhydryl groups of proteins (Gross *et al.*, 1970). Wu, Thurman and Bradshaw (1975), have shown a reduction in L - malate dehydrogenase activity in roots of the grass *Agrostis stolonifera* at up to 50  $\mu\text{M}$  copper, which was attributed to reduced protein synthesis; this effect was reduced in the case of copper-tolerant plants of the same species. In addition to effects on plant roots, excess copper may also reduce metabolic activity in the soil. Tyler (1974) showed that soils contaminated with copper and zinc had reduced urease and acid phosphate activity and a depressed respiration rate.

Other plant processes are also sensitive to copper. Cedeno-Maldonado, Swader and Heath (1972) have reported that copper (25  $\mu\text{M}$ ) inhibits photosynthetic electron transport in isolated chloroplasts. This may be due to an alteration in the structure of chlorophyll. Gross *et al.* (1970) observed that photosynthesis in the green alga *Chlorella sp.* was inhibited by copper, and coincident with this was a change in the absorption spectrum of the algal chlorophyll.

### **1.3 Root Uptake of Zinc by Plants**

Zinc is a micronutrient that is essential for a number of different plant functions, including disease resistance and the ability of the plant to resist a number of abiotic environmental stresses (e.g., salt stress, drought stress). Zinc is taken up into the plant primarily by the absorption of the zinc ion,  $\text{Zn}^{2+}$ , from the soil solution to the root (Kochian, 1994). Kochian (1994) studied  $\text{Zn}^{2+}$  uptake and presented data that indicates  $\text{Zn}^{2+}$  can be transported into root cells via an ion channel that also might transport some other mineral nutrients and heavy metals that exist as divalent cations, including copper and cadmium.

Zinc is an element of intermediate mobility. When zinc is supplied at luxury levels, it tends to accumulate in the root; however, if this level is not maintained, there is ultimately transport from root to shoot (Loneragan, 1977). Evidence for translocation of

zinc comes from the work of Massey and Loeffel (1967) who showed that zinc accumulation in the developing ear of maize, between tasselling and maturity, exceeded total uptake during that period by 50 %. The excess zinc came from the surrounding stem and leaf tissue.

### 1.3.1 Source of Zinc and Its Symptoms of Toxicity

Some soils contain abnormally high levels of zinc. This can be a geochemical phenomenon, for instance where the soil overlays zinc-rich minerals, or it may be a man-made condition. Examples of man-made conditions are spoil heaps at mining sites, sewage sludge often rich in heavy metals applied for agricultural purposes, or excess zinc oxysulphate persisting in the soil after use as a pesticide (Collins, 1981).

The general symptom of zinc toxicity is a retardation of growth, with plants being stunted and chlorotic. Takkar and Mann (1978) described zinc toxicity symptoms in wheat and maize: a light blue-green tinge develops at the leaf tips and spreads to the base, older leaves are chlorotic, finally the leaf dries out. Taylor *et al.*, (undated) described the most common zinc excess symptoms as red discoloration (especially of veins), young leaves chlorotic, leaves curl downwards and necrosis of shoot tips.

Takkar and Mann (1978) found that a soil level of 7 ppm zinc (DPTA-extractable) was toxic to wheat and 11 ppm zinc toxic to maize. The tissue levels at these soil levels were 60 and 81 ppm dry matter, respectively. Somewhat higher levels have been recorded by Beckett and Davis (1977) and Davis and Beckett (1978) for barley (*Hordeum vulgare*) and ryegrass (*Lolium perenne*) tissue. These authors observed tissue contents of zinc to vary considerably depending on the growth conditions, but found that the minimum tissue concentration necessary to cause toxic symptoms was relatively independent of growth conditions. Above the upper critical level of zinc the yield was found to decrease in approximate proportion to the logarithm of the tissue concentration.

### 1.3.2 Effect of Excess Zinc on Metabolic Processes

The mechanism of specific actions of elevated zinc on physiological processes is not clear, either it is direct or indirect. The inhibitory effect of zinc on phloem translocation was demonstrated by Samarkoon and Rauser (1979). They found that 0.2 mM zinc severely restricted carbohydrate translocation in *Phaseolus vulgaris*. Within 1-2 days following treatment transport to the sinks was drastically reduced, with a concomitant accumulation of sucrose, reducing sugars and starch in the primary leaves. The mechanism of this inhibition was not determined. Although zinc caused callose deposition on sieve plates this was not thought directly responsible (Peterson and Rauser, 1979).

The chlorotic response in zinc toxicity has frequently been attributed to an interference with iron metabolism, and indeed chlorosis can often be overcome, at least partially, by the addition of iron. However, the chlorosis is not associated with decreased leaf iron levels, and iron appears to retard the absorption and translocation of zinc (Rosen, Pike and Golden, 1977). Ambler, Brown and Gauch, (1970) produced evidence that high levels of zinc reduce the amount of reductant necessary for iron translocation by soybean (*Glycine max*) roots; addition of iron chelate overcame this problem.

There is a marked difference in susceptibility of species and genotypes of species to zinc toxicity. For the soybean (*Glycine max*) cultivars 'Wye' and 'York', grafting experiments have shown that tolerance to excessive zinc levels resides in the shoot genotype. The root stock genotype controlled zinc absorption and translocation. However the differences in yield due to susceptibility of the shoot genotype were not related to leaf zinc content (White, Chaney and Decker, 1979). Work by Symeonidis and Karataglis (1992) has shown the detrimental effects of zinc on both root and shoot growth and chlorophyll activity of *Holcus lanatus*.

Although there are considerable data on the effect of these metals on whole-plant physiology, little is known about their effects on plant reproductive tissues and the

reproductive potential of plants. Baloch (1994) found some evidence that *Arabidopsis* mutant sporophytes that are tolerant to high levels of aluminium, copper, cadmium and zinc also produce pollen gametophytes that are more tolerant to these metals *in vitro* and possibly *in vivo*.

#### **1.4 Ecological importance of soil pollution with copper and zinc.**

It is now well known that although among the heavy metals some, e.g. copper, zinc, are essential for plant growth, when present at elevated levels in soils, they are generally toxic and can ultimately cause the death of plants. The toxicity is mostly manifested in retardation or complete inhibition of root growth at higher metal levels (Baker, 1978).

That plants occur at all on soils with high or abnormal concentrations of metallic ions indicates that they must be adapted to the peculiar conditions of the site. Plants may avoid any toxicities by colonizing areas of lower metal concentration within the contaminated area or avoid, through some exclusion mechanism or selective uptake, excessive cellular concentrations of metallic ions (Antonovics, Bradshaw and Turner, 1971). However, in many instances, high concentrations of metals are found in the tissues of plants growing on contaminated soils and such plants are considered to be metal-tolerant (Bannister, 1976).

A special vegetation is found on the various ore outcrops or near metal smelters and among these plant species, ecotypes are known with a specific resistance to different heavy metals. Plants like *Agrostis capillaris* (= *A. tenuis*) and *Silene vulgaris* (= *S. cucubalus*) have been successful by changing their metabolism in such a way that they can grow and reproduce in metalliferous soils. *Agrostis stolonifera* is another example of such a plant which dominated copper-contaminated grassland near a metal refining industry in SW Lancashire which was established about 1900. Total soil copper level reached up to 4,000 ppm where in some places the vegetation has been totally destroyed

(Wu and Bradshaw, 1972). The authors found that there is a considerable increase in the occurrence of tolerance which supports the idea that selection of different genotypes for copper tolerance had taken place from an original non-tolerant population which gave rise to a tolerant subsequent generation. A study by Al-Hiyaly, McNeilly and Bradshaw (1988) showed the rapid evolution of zinc tolerance of *Agrostis capillaris* L. under a series of zinc-coated electricity pylons since less than 30 years ago. The zinc-contaminated soil beneath the pylons was sufficient to impose selective forces by which zinc-tolerant populations would develop in a replicated manner. High contamination by zinc imposes selection pressures sufficient to cause the preferential survival of zinc-tolerant genotypes and therefore build up localized tolerant populations. For such a situation to occur, appropriate species with the ability to evolve zinc tolerance must be present. Symeonidis, McNeilly and Bradshaw (1985) have shown that even within *A. capillaris*, a widespread colonizer of abandoned zinc mine wastes, the ability to respond to selection for tolerance to different metals, including zinc, was population dependent. There is variability in a population to respond to selection. Al-Hiyaly *et al.* (1988) also found that zinc toxicity found in such soils caused significant reduction in plant dry weight and tiller production of normal material of *A. capillaris*. Industrial pollution has also adverse effects on the physiology of pollen. Salgare and Palathingal (1993) conducted a pollen-culture study on *Peltophorum ferrugineum* Benth. They found that industrial pollution caused up to 70.14 and 50.54 % inhibition in the rate of pollen germination and pollen tube growth respectively.

Various studies on tolerance of pollen have shown that some plants are known to have become adapted to growing on soils with high heavy metal levels. Also, if there are observable effects on pollen germination or pollen tube growth that are correlated with heavy metal tolerance, these parameters could be used to select copper and zinc-tolerant plants for land reclamation schemes and, perhaps, as a bioassay for heavy metal levels in the soil.

## **1.5 Pollen**

Pollen transmits the male genetic material in sexual reproduction of all higher plants. The same pollen is also well suited as a research tool for studying many patterns of plant metabolism, genetics and physiology. Pollen development and activity are among the most sensitive indicators of adverse factors in the botanical environment (Stanley and Linsken, 1974). Environmental conditions can affect pollen characters that may, in turn, affect paternal success (Young and Stanton, 1990). For example, environmental conditions during pollen development have been shown to influence pollen germination (Schlichting, 1986), pollen viability (Sharma *et al.*, 1985) and pollen tube growth rates (Miller and Schunhorst, 1968).

### **1.5.1 Pollen Germination and Tube Growth**

Pollen germination and tube growth rate depend strongly on pollen quality. The quality of pollen itself is influenced by environmental conditions during pollen development such as temperature, air humidity and nutrient supply.

#### **Nutrient Supply**

Young and Stanton (1990) found that pollen produced under low nutrient conditions sired fewer seeds than pollen produced under better conditions when the two types were applied on a stigma together. This may be due to differential pollen tube growth rates - pollen tubes from low nutrient supply plants grow more slowly than pollen tubes from control plants and thus reach ovules later.

Pollen germination and tube growth were sensitive to heavy metal toxicity (Bhandal and Bala, 1989; Sawidis and Reiss, 1995). Bhandal and Bala (1989) observed complete

inhibition of pollen germination at different concentrations (between 100 - 200  $\mu\text{M}$ ) in most of the heavy metals (Cd, Ni, Cu, Pb and Zn) except Co. The inhibitory effect of different heavy metals salts except  $\text{Cu}^{2+}$  on pollen germination and tube growth was partially reversible. Inhibition of TTC reduction (tetrazolium chloride reduction) by heavy metals may reflect inhibition in pollen respiration, which consequently inhibits pollen germination and tube growth. Work by Sawidis and Reiss (1995) demonstrated the toxicity of  $\text{Cu}^{2+}$ , with 30 $\mu\text{M}$  showing the most toxic effect on pollen germination and causing drastically reduced average tube length. For  $\text{Zn}^{2+}$ , the most toxic effect on pollen germination and tube length was at 100 $\mu\text{M}$ . The measurements were done after 3 hours of incubation in the presence of metals, and beyond that time, the tubes showed an increasing tendency to burst.

### **Temperature**

Under normal conditions the pollen of most plants shows optimum germination between 20 °C - 30 °C, and the  $Q_{10}$  is approximately 2. Higher temperatures cause bursting of pollen and the pollen tubes show abnormal forms (Johri and Vasil, 1961). Low temperatures increase plant sterility by blocking pollen development, and in particular the stamens and pollen are more sensitive to temperature during development than other parts of the flower (Stanley and Linsken, 1974). Younger (1961) showed that low temperature resulted in pollen abortion and inhibition of stamen development in *Pennisetum clandestinum*, although stigma development continued normally at the same low temperature. Yamada and Hasegawa (1959) have shown that rice (*Oryza sativa*) plants grown with a root temperature of 28 °C yielded pollen with higher viability than plants grown with roots at 23 °C or 33 °C.

### 1.5.2 Pollen Viability

Pollen viability is generally considered to indicate the ability of the pollen grain to perform its function of delivering the sperm cells to the embryo sac following compatible pollination (Shivanna, Linskens and Cresti, 1991). Assessment of pollen viability is based on two tests: *in vitro* germination test and fluorochromatic reaction (FCR) test.

Studies on pollen viability with pollen vigour has been given little attention. It is expected that stored pollen or pollen subjected to environmental stresses, may exhibit reduction in vigour before pollen loses its ability to germinate (Shivanna *et al.*, 1991). In their earlier studies Shivanna and Cresti (1989) and Shivanna *et al.*(1990), found that high relative humidity and high temperature (38/45°C) stressed pollen of *Nicotiana tabacum*. They found that pollen vigour was significantly affected but not pollen viability. Pollen samples took longer to germinate (at 38°C) or failed to germinate (45°C) *in vitro*; they did germinate on the stigma but the pollen tubes took much longer to reach the ovary when compared to the controls.

A further investigation was carried out by Shivanna *et al.* (1991) on pollen viability and pollen vigour. They showed that stressed pollen exhibits loss of vigour before it loses the ability to germinate or to respond to the FCR test. These authors used the FCR test to assess pollen viability and an *in vitro* germination test was used to assess pollen vigour. They found that to assess the vigour of the pollen by *in vitro* germination test, the rate of *in vitro* germination is monitored over a period of time and then compared with the responses of the fresh pollen. However, it is argued that the time taken for pollen to germinate *in vitro* is generally longer than the time required for the germination of fresh pollen. Alternatively, pollen vigour can also be studied by carrying out *in vivo* pollinations and monitoring pollen tube growth in the pistil using aniline blue fluorescence.

Pollen grains are short-lived and sensitive to a number of factors. Pollen viability is also related to yield of crop plants. Apart from giving a measure of toxicity, the study

of pollen viability provides valuable understanding about its impact on yield (Handique and Baruah, 1995). Marcucci *et al.* (1983) have shown that reduction in yield is positively co-related with reduction in pollen viability caused by application of pesticides and fungicides at the time of flowering.

The loss of pollen viability in pollen of tomato (*Lycopersicon esculentum*), pea (*Pisum sativum*) and pigeon pea (*Cajanus cajan*), following treatment with heavy metals, is related to a high concentration of heavy metal and long duration of treatment (Handique and Baruah, 1995). These authors inferred that more heavy metal ions penetrated the pollen grains, resulting in inactivation of the enzyme dehydrogenase and consequent loss of viability. Bhandal and Bala (1989) observed negligible trinitrophenyl tetrazolium chloride (TTC) reduction activity, in cultured *Amaryllis vittata Ait* (*Amaryllidaceae*) with 150  $\mu\text{M}$   $\text{Cu}^{2+}$  containing medium, which indicates loss of pollen viability. Loss of viability during storage may be due to utilization of stored food materials, desiccation and inactivation of certain vital systems like the enzymes (Johri and Vasil, 1961).

## 1.6 Pollen Germination Medium

The requirements for pollen to germinate in artificial media varies depending on the plant species used. However, the basic and most common requirements are sucrose, boric acid and calcium.

The role of sugar in pollen germination is to serve as a nutritional source and osmoticum. The effect of boron on pollen germination and on elongation of tubes is much more marked than the effect of any known hormone, vitamin or other chemical. It promotes absorption of sugars and their metabolism by forming sugar-borate complexes, increases oxygen uptake and is involved in the synthesis of pectic materials required for the wall of the actively elongating pollen tube (Johri and Vasil, 1961). Calcium has also played an important role in pollen studies. Brewbaker and Kwack (1964) concluded that

calcium overcomes the population effect, promoting germination and elongation in all pollen types tested (over 100 species), both in culture and *in situ*. They also concluded that the calcium effect is dependent on the presence of a suitable osmotic milieu, oxygen and borate and is enhanced by methyl donors and other inorganic cations (especially Mg, K, Na and H). Brewbaker and Kwack (1963) recommended the broad based basal medium (10 % (w/v) sucrose, 100 ppm  $H_3BO_3$ ; 300 ppm  $Ca(NO_3)_2 \cdot 4H_2O$ ; 200 ppm  $MgSO_4 \cdot 7H_2O$ ; 100 ppm  $KNO_3$ ) which is widely used for experiment on many species.

Only in 32 % of the angiosperm families are some or all of the species characterized by tricellular pollen (Brewbaker, 1967). Tricellular pollen is difficult to germinate *in vitro*, when compared to bicellular pollen. They often demand water-restricting conditions (Bar-Shalom and Mattsson, 1977) or high sugar concentrations. Often pollen tubes are very short. They are short-lived whatever the conditions (Cerreau-Larrival and Challe, 1986).

*Brassica* species produce 3-celled pollen. There has been limited success of *in vitro* pollen germination and tube growth in 3-celled systems (Vasil, 1987), as also reported by earlier studies on *in vitro* pollen germination of *Brassica* as poor, inconsistent and often shows a genotypic response (Chiang, 1974a; Ferrari and Wallace, 1975). Roberts *et al.*, (1983) formulated media containing  $KNO_3$ ,  $CaCl_2 \cdot 6H_2O$  and  $H_3BO_3$  with high pH (8.0-9.0) and a high sucrose concentration (20 %). The addition of Tris has established an optimum pH and has been found to be effective in stimulating germination at all pH's. The "amine effect" observed on the assay has been interpreted as an artificial stimulation of grain metabolism in a liquid environment, which is not required in the natural situation. The assay produced good percentage germination (from 20 % to 80 %), but pollen tube growth was not satisfactory. The pollen tube growth increased with the lowering of sucrose level, but this decreased pollen germination.

Shivanna and Sawhney (1995) improved the medium of Robert's *et al.* by incorporating polyethylene glycol (PEG) 4000 in the medium and by lowering sucrose concentration (from 20 % to 5 or 10 %). They achieved high percentage germination and

high tube growth. In different concentrations of PEG (10,15 and 20 %) with 2.5 - 10 % sucrose, the maximum pollen tube growth in *Brassica napus* (cv. Wester) was obtained in media with 15 % PEG and 10 % sucrose in 2 hours.

In different concentrations of sucrose (2.5, 5, 10, 15 and 20 %) with 10 % and 15 % PEG, the maximum tube growth was obtained with 15 % PEG and 5 % sucrose in 4 hours. The optimal combination of sucrose and PEG varied from species to species; 10 % sucrose + 10 % PEG for *Brassica napus* var. ISN 706, 5 % sucrose + 20 % PEG for *Brassica juncea*, and 10 % sucrose + 15 % PEG for *Brassica campestris*. In all *Brassica* species tested, PEG not only promoted high pollen germination, it also increased the rate and duration of tube growth, with the exception of *Brassica oleracea* which recorded low pollen germination, that is, 20-30 % (Ferrari and Wallace, 1975). A study by Rihova *et al.* (1996) has shown that PEG promotes pollen germination and tube growth in potato. The best results were achieved with 20 % PEG 1000 and 2 % sucrose at 1.6 mM H<sub>3</sub>BO<sub>3</sub>. DumontBeBoux and von Aderkas (1997) found that the survival rate of pollen of Douglas-fir (*Pseudotsuga menziesii* (Mirb.) France) was best on PEG-glucose medium. They also showed that when PEG was the sole osmoticum, the pollen tubes grew longer and straighter, compared to in sucrose - where the pollen tubes were short and distorted.

### 1.7 *In vitro* versus *in vivo* pollen germination

*In vitro* experiments are an artificial reflection of what may happen *in vivo*. The findings obtained from *in vitro* might be comparable to that of *in vivo*, although there are drawbacks to *in vitro* tests that need to be carefully controlled, such as temperature, concentration of grains and substrate constituents that can affect pollen tube growth (Stone, Thomson and Dent-Acosta, 1995).

Most pollen tubes cultured *in vitro* stop growing before they reach the size normally attained in the style and the rate of tube growth is seldom as rapid as *in vivo*. This suggests that optimum growth conditions are not as often established in *in vitro*

media (Stanley and Linsken, 1974). In most species, the *in vitro* tube length may be 10 % of that of pollen tubes *in vivo*. This strongly supports the idea that pistil tissue provides a much more suitable milieu for the rapid growth of the pollen tube than that provided *in vitro*. However, in the case of *Tradescantia paludosa*, not only do pollen tubes reach the length of those produced in nature but these even grow at about same rate in liquid suspension culture as in the style and in most other respects their growth *in vitro* is comparable to that *in vivo* (Mascarenhas, 1966). Rate of pollen tube growth *in vivo* is in the range of 1500-3000  $\mu\text{m}/\text{h}$  in most species with a maximum of 7500  $\mu\text{m}/\text{h}$  (Buchholz and Blakeslee, 1927; Brewbaker and Kwack, 1964). Vasil (1960) reported rates in excess of 2000  $\mu\text{m}/\text{h}$  for pollen tubes of *Cucumis melo in vitro*. Hoekstra (1983) observed that some pollen tubes of *Geranium maculatum* reached the ovary within 30 minutes of germination. Their actual growth rate through the 4 mm style tissue is therefore at least 0.133 mm/min., fairly typical for trinucleate pollen.

The first stage in pollen germination is believed to involve the uptake of water by the pollen grains (Mascarenhas and Bell, 1969). The moisture of pollen for germination is provided by various stigmatic secretions, but in *Cruciferae*, because the stigmatic surface is covered by a thick layer of cuticle, this initial moisture is believed to be obtained from cuticular transpiration (Linskens and Kroh, 1970). The stigmatic surface in leguminous plants generally has an exudate which contains sugars, lipids, amino acids, phenolic compounds and proteins (Heslop-Harrison and Shivanna, 1977). The exudate helps to retain pollen on the stigmatic surface and acts as a medium for pollen grain germination and pollen tube growth (Konar and Linskens, 1966). Turano, Baird and Webster (1983) reported an exudate on the stigmatic surface of chick pea (*Cicer arietinum*) localized on the most distal cluster of papillate cells of the stigma.

In many of the angiosperm species pollen grains germinate in sugar solution. Sugar serves as chief nutritional sources as well as osmotic control *in vivo* or *in vitro*. Chiang (1974b) used 16 sugars as carbon sources for cabbage pollen germination. Only six; sucrose, raffinose, lactose, maltose, melizitose and trehalose supported germination. Sucrose produced the highest percent of germination, while raffinose produced the longer

pollen tubes. Rihova, Hrabetova and Tupy (1996) substituted sucrose with raffinose, maltose, glucose, galactose, fructose, or mannitol for *in vitro* pollen germination and tube growth in potato and found that they did not improve the medium for germination. Hrabetova and Tupy (1964) evaluated the effect of different sugar media on the growth of pollen from 49 species. They reported that the pollen tubes of 41 species had best growth on a sucrose medium. Pollen tubes of 7 species grew on a glucose medium, whereas pollen tubes of *Salix caprea* grew on a fructose medium. Pearson (1932) stated that cabbage pollen could germinate in 1 % (w/v) glucose but satisfactory germination could be achieved by supplementation of glucose with boric acid. Rashid, Illahi and Hussain (1985) achieved germination of pollen of *Juniperus sp* in a medium containing a mixture of 0.5 % boric acid solution and 5 % sucrose solution at 18 °C.

Boron is an essential element for normal development and growth of higher plants (Maze, 1915, Warrington, 1923). There have been studies showing high concentrations of boron in tissues of the stigma, style and the ovary which play an important role in fertilization. The stigma may contain 10 times the amount of boron than that found in pollen, where the boron content of pollen is 0.7µg/mg dry weight (Stanley, 1971). Chiang (1974b) used 16 combinations of H<sub>3</sub>BO<sub>3</sub> and CaCl<sub>2</sub>.2H<sub>2</sub>O, and found that maximum germination percentage was obtained by addition of 50 ppm and 100 ppm of boric acid and calcium respectively. A recent study by Fernando *et al.* (1997) found that lack of H<sub>3</sub>BO<sub>3</sub> inhibited pollen tube formation, whilst addition of H<sub>3</sub>BO<sub>3</sub> and Ca(NO<sub>3</sub>)<sub>2</sub> significantly increased pollen tube formation within one week in culture.

*In vitro* growth curves of pollen have been described as sigmoid for many species (Vasil, 1960, 1962). These are categorized in three phases. The first is a lag phase of slow growth, followed by rapid elongation period and finally a period of gradual decrease in growth resulting in stoppage of tube growth. Brewbaker and Majumder (1961) described how growth patterns of pollen populations show a brief lag phase, a linear growth phase and a final levelling out phase. They emphasised that under optimal conditions individual pollen tubes grow at a linear rate. Ottaviano, Sari-Gorla and Mulcahy (1980) stated that

Mulcahy (1980) stated that the speed of pollen tube growth is positively correlated with the quality of the resultant sporophytic generation.

*In vivo* tests are doubtless the most exacting available for determining pollen viability, with seed set or pollen tube growth being easily measurable variables. However, the test itself occupies time and stigmatic surfaces potentially limiting sample sizes. These drawbacks can be overcome by *in vitro* tests (Stanley and Linsken, 1974).

## 1.8 Storage of Pollen

The longevity of pollen of different species varies from minutes to years depending on the taxonomic status of the plant and on the abiotic environmental conditions. In most plants pollen loses viability soon after dehiscence of the anthers, especially where temperature and humidity are unusually high. Occasionally viability may be retained for some time, even under natural conditions (Johri and Vasil, 1961).

For this, special storage conditions are needed to preserve the viability and fertilizing ability of pollen for a long period. Cryopreservation seems to be the most efficient method for the long-term preservation (up to 10 years) of partly dehydrated pollen grains (Barnabas and Kovacs, 1994). Rajasekharan and Ganeshan (1994) also pointed out that cryo-stored pollen of four rose cultivars retained its ability to fertilize and produce seeds which would enable breeders to pollinate at any time of the year at any location. For short-term pollen storage, using a desiccator placed at low temperature also retained pollen viability, for example, *Verticordia sp.* pollen retained 75-80% viability at -196 °C, and even when stored at room temperature for 6 months maintained a viability of 50% (Tyagi, Considine and McComb, 1992). Shivanna *et al.* (1991) found that storage of *Nicotiana tabacum* pollen and *Agave* species pollen under laboratory conditions did not affect FCR score but significantly delayed germination.

A major source of difficulty to those engaged in the storage of pollen is the erratic germination of stored samples. Quite often even if pollen fails to germinate in one or two previous tests, it may give a high percentage in subsequent tests. In some other cases, the percentage of germination after storage for a few days is higher than that of fresh pollen (Johri and Vasil, 1961). Such irregularities are generally found when the pollen is collected from apparently equally mature flowers on different days or in different, though neighbouring, localities, and even when it is taken from flowers at the same age in a given locality and at about the same time (Holman and Brubaker, 1926).

### 1.9 Cytoplasmic Streaming

The growth of pollen tube *in vitro* is characterised by a typical sigmoid curve which illustrates the first phase of slow growth, followed by a rapid elongation period and finally a period of gradual decrease in growth resulting in stoppage of tube growth. The rate of cytoplasmic streaming varies from plant to plant and is directly proportional to the rate of elongation of the tube (Vasil, 1960a).

Cytoplasmic streaming begins within a few minutes after the pollen is sown in the culture medium or lands on a stigma and continues until the pollen tube has ceased to elongate. The rate of movement (*in vitro*) is slow at the beginning, gradually increases and comes to a peak slightly before the middle of the growth period, and then gradually declines until the pollen tube stops elongating. Occasionally, however, the streaming movement may continue for some time even after the tube has ceased to elongate. In an actively growing pollen tube, the rate of streaming is rather slow near the pollen-grain end, is fairly rapid in the rest of the tube, and there is practically no streaming in the extreme end (Johri and Vasil, 1961). Decline in cytoplasmic streaming rates may be a sensitive indicator of the toxicity of xenobiotics.

## 1.10 Objectives of the present study

The primary objective of this study was to determine the effects of heavy metals, specifically copper and zinc, on the viability of the male gametophyte *in vitro* and *in vivo* in flowering plants. In addition, experiments were conducted to determine optimum conditions and essential controls for the potential use of pollen gametophytes in monitoring heavy metal contamination.

Experiments were designed to test the following hypotheses:

That:

- (1) Heavy metals affect pollen viability.
- (2) Heavy metals affect pollen germination.
- (3) Pollen tube length *in vitro* is affected by the presence of heavy metals in the germination medium.
- (4) The general pattern of effects observed relate to flowering plants in general, and are not species-specific.
- (5) Effects of heavy metals on pollen germination and tube growth *in vitro* are an indication of similar effects *in vivo*, during growth of pollen in the gynecium.
- (6) The presence of heavy metals in a pollen germination medium can be detected through measuring the rate of cytoplasmic streaming in pollen tubes.

The outcomes of these experiments may then be used to devise a set of criteria for the use of flowering plant gametophytes as an assay for the presence of toxic heavy metals in the gametophytes.

## Chapter II

### Materials and methods

#### 2.1 Research Plant Material

The following plant species were used for experimentation:

Family name: *Crassulaceae*

Genus name: *Kalanchoe fedtschenkoi*

Origin: Native to arid areas in the Old World tropics.

Source: Flowers obtained from plants growing in the Arid House in Durham University Botanic Garden.

Characteristics: A perennial dicotyledenous plant which grew well and flowered during the winter season, and therefore pollen was available for experimentation.

Family name: *Liliaceae*

Genus name: *Hippeastrum* sp.

Origin : Native to tropical America.

Source: Bulbs purchased from a retail outlet, Woolworths plc.

Characteristics: A perennial bulb forming a monocotyledenous plant with very large pollen tube which is suitable for analysing cytoplasmic streaming rate.

Family: *Cruciferae*

Genus : *Brassica rapa*:

Origin: Native to temperate regions of Eurasia.

Source: Rapid cycling genotypes obtained originally from the University of Wisconsin and maintained at University of Durham Department of Biological Sciences.

Characteristics: It is a rapid-cycling annual dicot. plant with a rapid generation time, producing large numbers of flowers. It is available in genetically-defined stocks. The rapid life cycle is between 35 to 40 days, seed-to-seed, when grown under continuous cool white fluorescent light. The plants can be sown at high densities under fluorescent

lighting, and produce flowers 14 days after planting. Fertilisation occurs within 24 hours of pollination and pods visibly swell 3 to 5 days after pollination.

The experimental plants were chosen on the basis of:

- i) well-separated taxonomy which would allow investigation of reproductive characteristics over a wide taxonomic range;
- ii) availability of plants;
- iii) suitability for *in vivo* assay;
- iv) size of the pollen grains.

## 2.2 Germination medium

Two *in vitro* germination media were used:

1. A simple germination medium was used which contains 50 ppm  $\text{H}_3\text{BO}_3$  and 2000 ppm  $\text{Ca}(\text{NO}_3)_2$ .
2. Roberts *et al.* (1983) germination medium was used as recommended by Shivanna and Sawhney (1995). They used Roberts *et al.* (1983) culture medium and added PEG 4000 to it. The details are listed in the table 2.1

Table 2.1 : Composition of Roberts *et al.* (1983) culture medium

Name of salt	Concentration ( $\text{mg l}^{-1}$ )
$\text{H}_3\text{BO}_3$	10
$\text{KNO}_3$	100
$\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$	362
Tris	80

Table 2.2: List of the chemicals used in experimentation.

Name of chemical	Name of supplier
H <sub>3</sub> BO <sub>3</sub>	BDH Chemical Ltd.
ZnSO <sub>4</sub> .7H <sub>2</sub> O	BDH
MgSO <sub>4</sub> .7H <sub>2</sub> O	BDH
NaH <sub>2</sub> PO <sub>4</sub> .2H <sub>2</sub> O	BDH
NaCl	BDH
Na <sub>2</sub> MoO <sub>4</sub> .2H <sub>2</sub> O	BDH
NaOH	BDH
Ethanol	BDH
Ca(NO <sub>3</sub> ) <sub>2</sub>	BDH Laboratory supplies
CaCl <sub>2</sub> .6H <sub>2</sub> O	BDH
KNO <sub>3</sub>	BDH
NH <sub>4</sub> NO <sub>3</sub>	BDH
K <sub>2</sub> SO <sub>4</sub>	BDH
MnSO <sub>4</sub> .4H <sub>2</sub> O	BDH
Tris	Sigma Chemical Co.
Sucrose	Sigma
CuSO <sub>4</sub> .5H <sub>2</sub> O	Sigma
Fluorescein diacetate	Sigma
Aniline blue	Sigma
Fe EDTA	Sigma

### 2.3 Microscope specification

A Nikon Diaphot-TMD inverted microscope with TMD-EF epifluorescence provided with violet (V), blue violet (BV), ultra-violet (UV) excitation filter cassettes, was used during the studies.

### 2.4 Image Analyser

The microscope mentioned above was coupled with a Micro Scale TM/TC manufactured by Image Analysis systems (Digithurst Ltd., England).

### 2.4.1 Use of Image Analyser

Images of pollen and pollen tubes were grabbed on a computer screen and either their dimensions measured or numbers counted. Pollen germination was counted and pollen tubes were measured using the dimension function on the microscale image analyser. The functions of microscale image analyser include:

- (a) Calibrate function:- providing operations that prepare the image for measurements, by setting calibration scales and by manually or automatically picking out the features of the interest to be analysed for instance, colour threshold, setting scale, region.
- (b) Dimension function:- Allows measurement of normal (straight) and polylines (curves).
- (c) Scan objects function:- Causes microscale image analyser to search, detect and measure all discrete objects in the current threshold region of interest.

### 2.5 Growth-room conditions

*Brassica rapa* plants were grown in a growth chamber, at a constant temperature of 25 °C, under constant illumination from Mercury vapour lamp (Philips, UK Ltd.) with light intensity of 330  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (PAR 400-700 nm). This was to induce flowering throughout the year. Seeds were planted in cocofibre compost in pots perforated from the bottom. The pots were placed in the trays and watered as frequently as necessary. After flowering, flowers were used for these studies.

*Hippeastrum* bulbs were grown in cocofibre compost at room temperature (20 °C).

## **2.6 Glasshouse conditions**

The glasshouse ambient temperature was 20 °C, with sixteen hour day-length.

## **2.7 Formulating pollen germination medium for *Kalanchoe fedtschenkoi***

### **2.7.1 Flower collection**

Freshly opened flowers were collected from the plants grown in the Arid glass house of the Botanical Garden of University of Durham in January, 1998. The pollen from the fresh flowers was collected gently using a pair of forceps.

### **2.7.2 Simple germination medium**

In initial experiment, the best germination conditions for *Kalanchoe fedtschenkoi* were sought. For this, the following simple germination medium was used for pollen germination and growth:

2000 ppm  $\text{Ca}(\text{NO}_3)_2$

50 ppm  $\text{H}_3\text{BO}_3$

Three media were prepared containing the above concentrations of  $\text{Ca}(\text{NO}_3)_2$  and  $\text{H}_3\text{BO}_3$  with the addition of sucrose to give a final concentrations of 5, 10 or 20 % sucrose in the medium.

### **2.7.3 Sample preparations**

Pollen grains were transferred into Petri dishes, onto the porous cellophane surface on filter paper, kept moist with the relevant media.

### **2.7.4 Incubation**

Samples were incubated in the simple germination medium at laboratory temperature for 1 hour, 1.5 hours and 2 hours.

### **2.7.5 Data Recording**

In each sample, 5 microscopic field views were randomly selected manually. In each field view 8 readings were recorded for pollen tube length (PTL) and one reading for percent of germination (%G). Measurements were made randomly, with the help of a microscope equipped with a computer image analysis system (see Sections 2.3 and 2.4).

### **2.7.6 Pollen germination assessment**

A pollen grain was considered as germinated if a tube appeared equal to, or greater than the pollen grain diameter. The total number of grains observed in a field was compared to the number of grains producing tubes (Stanley and Linskens, 1974).

$$\text{Pollen germination (\%)} = \frac{\text{Pollen germinated}}{\text{Total no. of grains observed}} \times 100$$

### **2.7.7 Germination medium of Roberts *et al.* (1983).**

In order to identify the best germination medium for *Kalanchoe fedtschenkoi*, three media containing H<sub>3</sub>BO<sub>3</sub>, CaCl<sub>2</sub>, KNO<sub>3</sub> plus Tris and either of 10 %, 15 %, or 20 % sucrose were prepared. Another three media were prepared containing H<sub>3</sub>BO<sub>3</sub>, CaCl<sub>2</sub>, KNO<sub>3</sub> and Tris at the same three concentrations of sucrose plus 10 % Polyethylene glycol (PEG). All the media were maintained at pH 6.0. From preliminary observations, it was revealed that 15 % sucrose gave maximal percent germination and pollen tube length. Therefore this sucrose concentration was used subsequently in all media.

#### **2.7.7.1 Sample preparation**

Three replicates of each medium, making a total of 36 samples were prepared. The fresh pollen collected was transferred onto the cellophane surface already saturated with the relevant media on filter paper in the Petri dishes.

#### **2.7.7.2. Incubation**

The samples were incubated at 1 hour, 2 hours and 3 hours at laboratory temperature.

#### **2.7.7.3 Effect of temperature on pollen germination and growth**

To identify the best temperature on pollen germination and growth, two temperatures 20 °C and 25 °C were chosen. The pollen grains were incubated in the controlled environment rooms maintained at these chosen temperatures.

#### **2.7.7.4 Effect of heavy metals on pollen germination and growth**

To evaluate the effect of heavy metals on the pollen germination and pollen tube growth,  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  was chosen for study at the concentration levels of 10, 20, 30, 40, and 50 ppm. The pH was at pH 6.0.

Three replicates of the medium at each concentration and controls, making a total of 54 samples were prepared. The pollen was transferred to cellophane as described above. The samples were incubated for 1, 2, and 3 hours at 20 °C and observations were recorded as previously mentioned.

#### **2.8 The response of different genotypes of the same species to the composition of the germination medium.**

To study the response of different genotypes towards Roberts *et al.* (1983) medium for *Brassica rapa* pollen, *self-compatible* and *dwf2* cultivars were used. Three pollen samples for each genotype were prepared with 15 % sucrose incorporated into the medium of Roberts *et al.* (1983) at pH 8.0. Samples were incubated for 1 hour, 2 hours and 3 hours. Pollen germination and growth data were recorded at each time point as per the procedure described earlier (see section 2.7.5 and 2.7.6).

#### **2.9 Pollen viability test**

A pollen viability test was carried out as follows: fresh pollen was suspended in 50 % (w/v) sucrose on a microscope slide. A drop of fluorescein diacetate ( $1 \text{ mg ml}^{-1}$ ) dissolved in 70 % ethanol was added to the specimen, then covered with a cover slip. The specimen was left for 3 minutes at laboratory temperature before examining under a fluorescence microscope, using the blue excitation filter. Viable pollen fluoresced bright

green. For each of the assays, 10 observations were taken after collecting pollen from different inflorescences. The percentage of viable pollen grains in each sample was calculated as the number of viable pollen against total number of pollen grains under a field view.

## **2.10 Formulating the pollen germination medium for *Brassica rapa*, *dwf2*.**

In order to find the best germinating medium for pollen of *Brassica rapa dwf2*, Roberts *et al.* (1983) germinating media were prepared with the following:

- 20 % sucrose
- 20 % sucrose plus 10 % PEG
- 20 % sucrose plus 15 % PEG
- 15 % sucrose
- 15 % sucrose plus 10 % PEG
- 15 % sucrose plus 15 % PEG
- 10 % sucrose
- 10 % sucrose plus 10 % PEG
- 10 % sucrose plus 15 % PEG

All the above media were maintained at pH 8.0 as described earlier.

### **2.10.1 Plant material**

Seeds of *Brassica rapa* were planted in pots with cocofibre compost medium and raised to flowering in the glass house of the Botanical Garden of University of Durham, at an ambient temperature of 20 °C in February, 1998 (see Plate 2.1).

### **2.10.2 Flower collection**

Fresh flowers which opened in the morning were excised at 10 am.

### **2.10.3 Sample preparation**

Three replicates were prepared for each medium, making a total of 81 samples. The pollen grains were transferred onto the cellophane surface already saturated with the relevant media on filter paper in the Petri dishes.

### **2.10.4 Effect of heavy metals on pollen germination and tube growth**

To evaluate the effect of heavy metals on the pollen germination and pollen tube,  $ZnSO_4 \cdot 7H_2O$  at concentrations of 20, 40, and 60 ppm and  $CuSO_4 \cdot 5H_2O$  at concentrations of 2, 4, and 6 ppm were chosen for study. Copper was found to be lethal to pollen of *Arabidopsis thaliana* above 10 ppm (Baloch, 1994). The pH was maintained at pH 8.0 as previously mentioned.

Three replicates of each medium with controls, making a total of 72 samples, were prepared. The fresh pollen was germinated as described above. The samples were incubated at 20 °C for 1 hour, 2 hours and 3 hours. Observations were recorded as per the procedure previously described.

### **2.11 *In vivo* pollen tube growth**

After stigma maturation, five flowers were hand-pollinated for each incubation period and the time noted. The incubation periods were of 2, 4, 6, 8, 24 and 48 hours.



Plate 2.1. *Brassica rapa* dwf2 plant grown in the glasshouse at the Botanic Garden, University of Durham. Inset shows close-up of flower.

After each incubation period at 20 °C in the glass house at the Botanical Garden, University of Durham, the flowers were excised from the mother plants. Floral parts were removed carefully with forceps and the gynaecial portions were retained in microcentrifuge tubes for staining.

### **2.12 Staining of the pistil**

Before staining the pistil, it was first fixed in 100 % ethanol solution. Then the pistil was transferred to a microcentrifuge tube containing 1 M NaOH solution kept in a water bath at 60 °C for 30 minutes to soften the cell walls. After hydrolysis, the pistil was transferred in decolourised aniline blue (0.5 % aniline blue in  $K_3PO_4 \cdot H_2O$ ) for 5 minutes to reveal the presence of callose. The pistil was then mounted on a glass slide with a drop of the decolourised aniline blue and covered with a cover slip. The pistil was squashed gently under a cover slip before examining the specimen under the fluorescence microscope, using a violet excitation filter. The measurements of pollen tubes running from stigmas to the pistil were made manually with the eye piece graticule fitted to the microscope.

### **2.13 Water culture medium used in plant growth.**

The water culture medium used was the Long Ashton nutrient solution. This standard complete nutrient solution used at Long Ashton was derived from nutrient solutions first used to grow fruit trees. The complete nutrient produces vigorous growth and good yields in a wide range of crops and no attempt has been made to develop numerous "optimum" complete nutrient solutions to suit the various needs of different crops. Long Ashton nutrient solution is said to be suitable under a variety of environmental conditions in water media and for numerous crops which include vegetables, trees of tropical and temperate species (Hewitt, 1966).

### **2.13.1 Plant material**

Seeds were sterilised by swirling the seeds in 5 % sodium hypochlorite solution for 3 to 5 minutes. The seeds were then washed in distilled water three times. Seed was sown on filter paper placed in a Petri dish. On average 30 seeds were sown in a Petri dish. The seeds were left in a dark room with available water sprays. After 2 days, germinated seeds were transferred to a growth room at 20 °C. Seeds were watered with distilled water every morning.

### **2.13.2 Long Ashton nutrient solution**

The ingredients of the Long Ashton complete nutrient solution in which plants of *Brassica rapa dwf2* were grown are listed in Table 2.3.

### **2.13.3 Water culture preparation**

After 7 days of growth on filter papers in Petri dishes, seedlings were transferred and grown in 500 ml capacity glass pots, containing 1/4-strength Long Ashton nutrient solution. The plants were first grown in 0 ppm (no added metals) as a control for 3 to 4 days, for the young plants to stabilise with the nutrient solution. The plants were then grown in solutions containing  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  at concentrations of 20, 40 or 60 ppm, and in  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  at concentrations of 2, 4, and 6 ppm. The plants were grown in a plant growth chamber at 25°C with 8 hours per day of light under fluorescent lighting ( $65 \mu\text{mol m}^{-2} \text{s}^{-1}$ ; PAR). All nutrient solutions were changed twice weekly (see Plate 2.2).

Table 2.3 Long Ashton nutrient solution

Salt	Concentration (%w/v)	Stock solution (g/l)	Volume of stock (ml) for 1 l of full strength LA	Volume of stock (ml) for 10 l of 1/4 strength LA	Milligram equivalent per litre obtained when 1 ml stock is diluted to 1 l	Final milliMolar (mM) conc. of compound after ten fold dilution to full strength LA
MgSO <sub>4</sub> .7H <sub>2</sub> O Magnesium sulphate. 7-hydrate	18.4	184	1	2.5	Mg <sup>2+</sup> 1.5 SO <sup>2-</sup> 1.5	0.75
NaH <sub>2</sub> PO <sub>4</sub> .2H <sub>2</sub> O sodium di-hydrogen orthophosphate	20.8	208	1	2.5	Na <sup>+</sup> 1.33 PO <sup>3-</sup> 1.33	1.33
Fe EDTA Ethylene diaminetetra- -acetic acid ferric	3.73	37.3	1	2.5		
NH <sub>4</sub> NO <sub>3</sub> Ammonium nitrate	16	160	1	2.5	NH <sup>4+</sup> 2 NO <sup>3-</sup> 2	2.0
K <sub>2</sub> SO <sub>4</sub> Potassium sulphate	8.7	87	1	2.5	K <sup>+</sup> 1 SO <sub>4</sub> <sup>2-</sup> 1	0.5
CaCl <sub>2</sub> .6H <sub>2</sub> O Calcium chloride 6-hydrate	43.4	438	1	2.5	Ca <sup>2+</sup> 4 Cl <sup>-</sup> 4	2.0
Monosodium complex						
					Milligram equivalents per litre or mM. Concentration produced when diluted at a rate of 0.1 ml/l	
MnSO <sub>4</sub> .4H <sub>2</sub> O Manganous sulphate 4-hydrate	2.23	22.3	0.1	0.25	Mn <sup>2+</sup> 0.02	mg. equil./l
CuSO <sub>4</sub> .5H <sub>2</sub> O Copper sulphate	0.25	2.5	0.1	0.25	Cu <sup>2+</sup> 0.002	mg. equil./l
ZnSO <sub>4</sub> .7H <sub>2</sub> O Zinc sulphate 7-hydrate	0.29	2.9	0.1	0.25	Zn <sup>2+</sup> 0.002	mg. equil./l
H <sub>3</sub> BO <sub>3</sub> Boric acid	3.1	31	0.1	0.25	B 0.05	mM
NaCl Sodium chloride	5.85	58.5	0.1	0.25	Cl <sup>-</sup> 0.1	mg. equil./l
Na <sub>2</sub> MoO <sub>4</sub> . 2H <sub>2</sub> O	0.12	1.2	0.1	0.25	Mo 0.0005	mM

#### **2.13.4 Effect of heavy metals on pollen viability**

Fresh, opened flowers were excised in the morning from plants cultured in solutions containing heavy metals. The procedure for assessing pollen viability was as described earlier (see section 2.9).

#### **2.13.5 Effect of heavy metals on pollen tube growth**

Flowers from plants grown in media containing heavy metals were hand-pollinated and the time noted. After a 6-hour incubation period at 25 °C in the growth room, the flowers were excised from the mother plants. From the preliminary observations, it was revealed that after 6 hours, the pollen tube had reached the ovule in the ovary.

Floral parts were removed carefully with forceps and the gynaecial portions were retained in microcentrifuge tubes for staining. The procedure for staining and measurements were described earlier (see section 2.12).

### **2.14 Rate of cytoplasmic streaming**

#### **2.14.1 Plant material**

*Hippeastrum* was used to study the cytoplasmic streaming rate because of its large pollen tube. For this study, two cultivars were used: *Hippeastrum* hybrids cv. *Belinda* and cv. *Appleblossom*.



Plate 2.2. *Brassica rapa* dwarf2 grown in hydroponics in the growth chamber.

### 2.14.2 Germination medium

To study the cytoplasmic streaming rate, the following germination medium was used: 20 % sucrose; 2000 ppm  $\text{Ca}(\text{NO}_3)_2$  and 50 ppm  $\text{H}_3\text{BO}_3$ . In an initial study, utilising a germination medium with 10 % sucrose, the pollen germinated but the pollen tubes burst.

### 2.14.3 Sample preparation

Three drops of germinating medium were placed onto a glass slide. Pollen was tapped onto the drop from a pair of forceps. The glass slide was placed on moistened filter paper on the Petri dish to reduce evaporation of the germinating medium. The specimen was covered by a Petri dish lid at laboratory temperature.

### 2.14.4 Data Recording

The pollen was incubated in the germination medium for 3 hours which was long enough to allow for sufficient growth of the pollen tubes to allow observation of a reasonable rate of movement of granules in the cytoplasm. The length of the pollen tubes was measured using the dimension and calibration function on the Microscale image analyser.

Calibration of the microscopic scale was set up as follows, before measuring the movement of granules along a specified distance:

The magnification of eye piece = 15X

The magnification of objective piece = 40X

The scale set between the eyepiece graticule and the microscopic scale : 40 units = 100 $\mu\text{m}$

$$\begin{aligned} 1 \text{ unit} &= \frac{100}{40} \\ &= 2.5\mu\text{m} \end{aligned}$$

A granule in the cytoplasm was selected to move to a distance of 25 units on the eye piece graticule which is equivalent to 62.5  $\mu\text{m}$ . The time taken for the granule to travel along this distance was recorded in seconds.

#### **2.14.5 Effect of heavy metals on cytoplasmic streaming**

To evaluate the effect of heavy metals on cytoplasmic streaming in the two *Hippeastrum* hybrids, the pollen grains were germinated in two concentrations of  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ : 50 ppm and 100 ppm. Measurements and data recording for cytoplasmic streaming were similar to those made previously (see section 2.14.4).

#### **2.14.6 Effect of heavy metals on the pollen tube growth length**

Apart from evaluating the effects of heavy metals on cytoplasmic streaming, pollen tube growth length of the two hybrids in  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  was also compared. Both samples were incubated for 5 hours. The pollen tube growth length of cv. Appleblossom was also assessed over time. Measurements and data recording were as described earlier (see section 2.14.4).

## Chapter III

### RESULTS

#### 3.1 Pollen germination and pollen tube growth length *in vitro* of *Kalanchoe fedtschenkoi*

##### 3.1.1 Identifying the best germination medium

In the initial experiment to find the best germination medium, simple germination media were used with three different concentrations of sucrose solutions (Fig. 3.1). Sucrose concentration in the germination medium significantly affected the percentage of pollen germination ( $p \leq 0.01$ ) with a significant ( $p \leq 0.05$ ) interaction of sucrose concentration with time. (Full statistical details for each experimentation are given in Appendix 1). Two concentrations of 5 % sucrose and 10 % sucrose showed increased percentage of pollen germination over time, with the latter giving the highest pollen percentage germination (40 %) (Fig. 3.1 a). Effects of sucrose concentrations upon pollen tube length were less marked ( $p \leq 0.05$ ), with no significant interaction term. A concentration medium of 20 % sucrose inhibited pollen germination and reduced the pollen tube length (Fig. 3.1 b).

Roberts *et al.* (1983) germination medium, with the addition of PEG as suggested by Shivanna and Sawhney (1991) was also used. Addition of 10 % PEG had no significant effect on %PG in 20 % sucrose over the 3 hour experimental period (Fig. 3.2 a). A similar overall outcome was also observed in the 15 % sucrose and 10 % PEG combination (Fig. 3.2 c). At 10 % sucrose in the germinating medium, addition of 10 % PEG substantially increased %PG over the initial 1-2 hours of the germinating period.

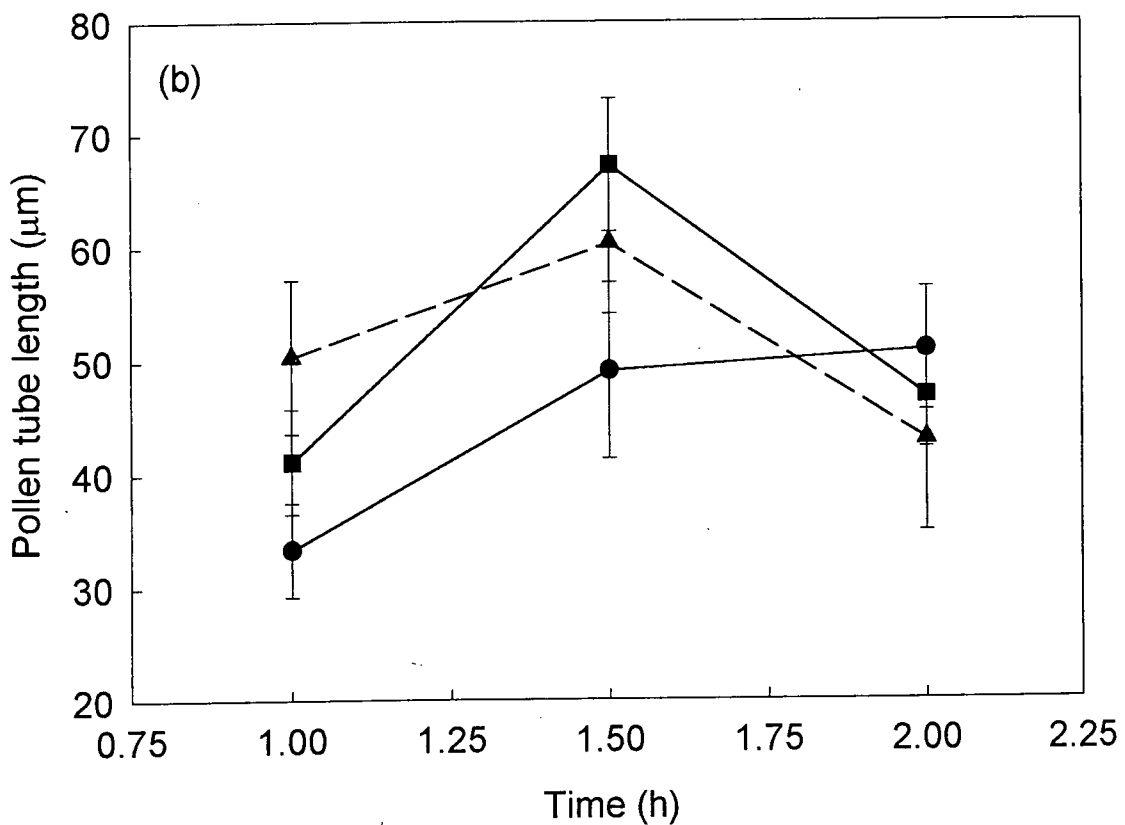
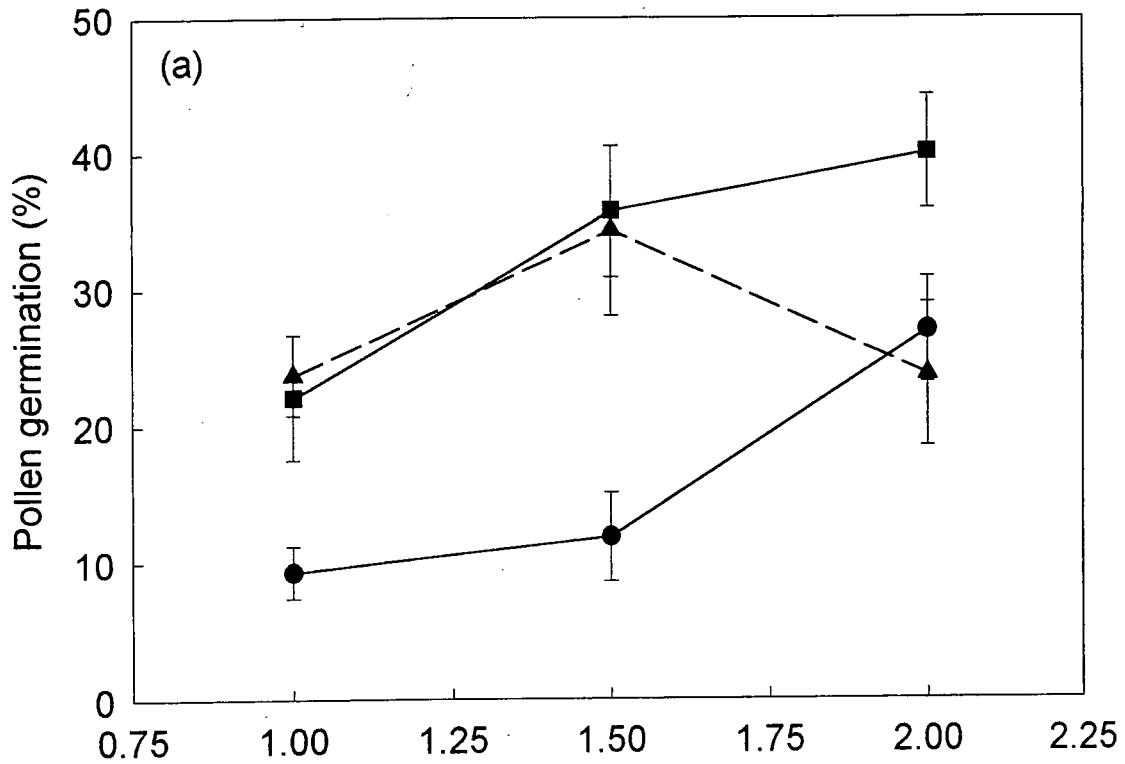


Figure 3.1. Percent pollen germination and pollen tube length of *Kalanchoe fedstchenkoi* in different sucrose concentrations in simple germinating medium. ●, 5 % sucrose; ■, 10 % sucrose; ▲, 20 % sucrose. Points represent separate batch samples of pollen. Data represent the mean  $\pm$  one SE of 40 separate determinations.

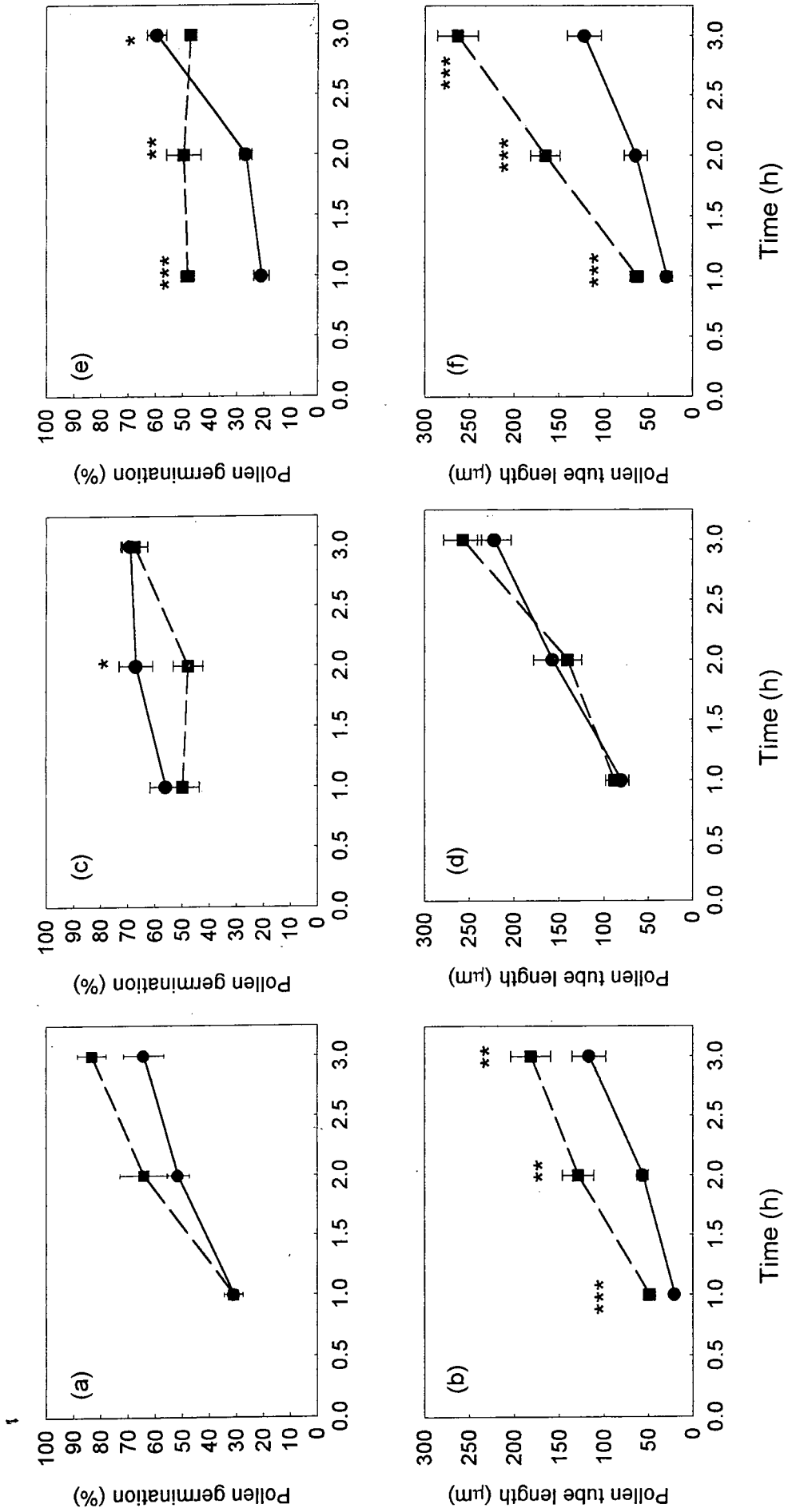


Figure 3.2. Percent pollen germination and pollen tube length of *Kalanchoe fedtschenkoi* in Robert's *et al.* germinating medium containing different concentrations of sucrose and PEG. All solutions were adjusted to pH 6.0. (a,b) ● = 20 % sucrose, ■ = 20 % sucrose plus 10 % PEG. (c,d) ● = 15 % sucrose, ■ = 15 % sucrose plus 10 % PEG. (e,f) ● = 10 % sucrose, ■ = 10 % sucrose plus 10 % PEG. Data represent the mean  $\pm$  one SE of three replicates.

However, after 3 hours, this effect was no longer seen, with %PG being higher ( $p \leq 0.05$ ) in the sucrose treatment alone (Fig. 3.2 e). Whilst having no significant effect on %PG in the 20 % sucrose treatment, addition of 10 % PEG significantly increased the PTL attained over the entire 3 hour experimental period (Fig. 3.2 b). However, no effect of PEG was observed in the 15 % sucrose treatment (Fig. 3.2 d). The greatest effect of PEG in enhancing PTL was seen in the 10 % sucrose treatment where PTL was increased by 54 % after 3 hours (Fig. 3.2 f).

### **3.1.2 Effect of temperature on pollen germination and tube length**

To identify the standard temperature to be used throughout the experiments, two test temperatures were chosen, 20 °C and 25 °C. Fig. 3.3 shows that a 5 °C temperature difference (20 - 25°C) had no significant effect in %PG (Fig. 3.3 a). However, there was a transient, small effect on PTL with a significant increase in PTL at 2 hours ( $p \leq 0.01$ ). After 3 hours, this effect was lost (Fig. 3.3 b). The 5°C temperature difference had little effect, thus the lower temperature (20°C) was chosen for use in further experiments.

### **3.1.3 Effect of heavy metals on pollen germination and tube growth.**

To study the effects of heavy metals on pollen germination and tube growth, five levels of zinc sulphate 10, 20, 30, 40, and 50 ppm were added to the germinating medium (Fig. 3.4). At low concentrations of Zn (10 ppm and 20 ppm) these treatments had a significant effect on %PG at initial germination, but later, there was no significant effect on %PG over time where the values returned similar to that of the control. There was a slower initial pollen germination rate, but later, this rate recovered (Fig. 3.4 b). A Zn concentration of 30 ppm, had no significant effect at all on %PG. However, in higher concentrations of Zn at 40 ppm and 50 ppm (Fig. 3.4 d, e), %PG was significantly

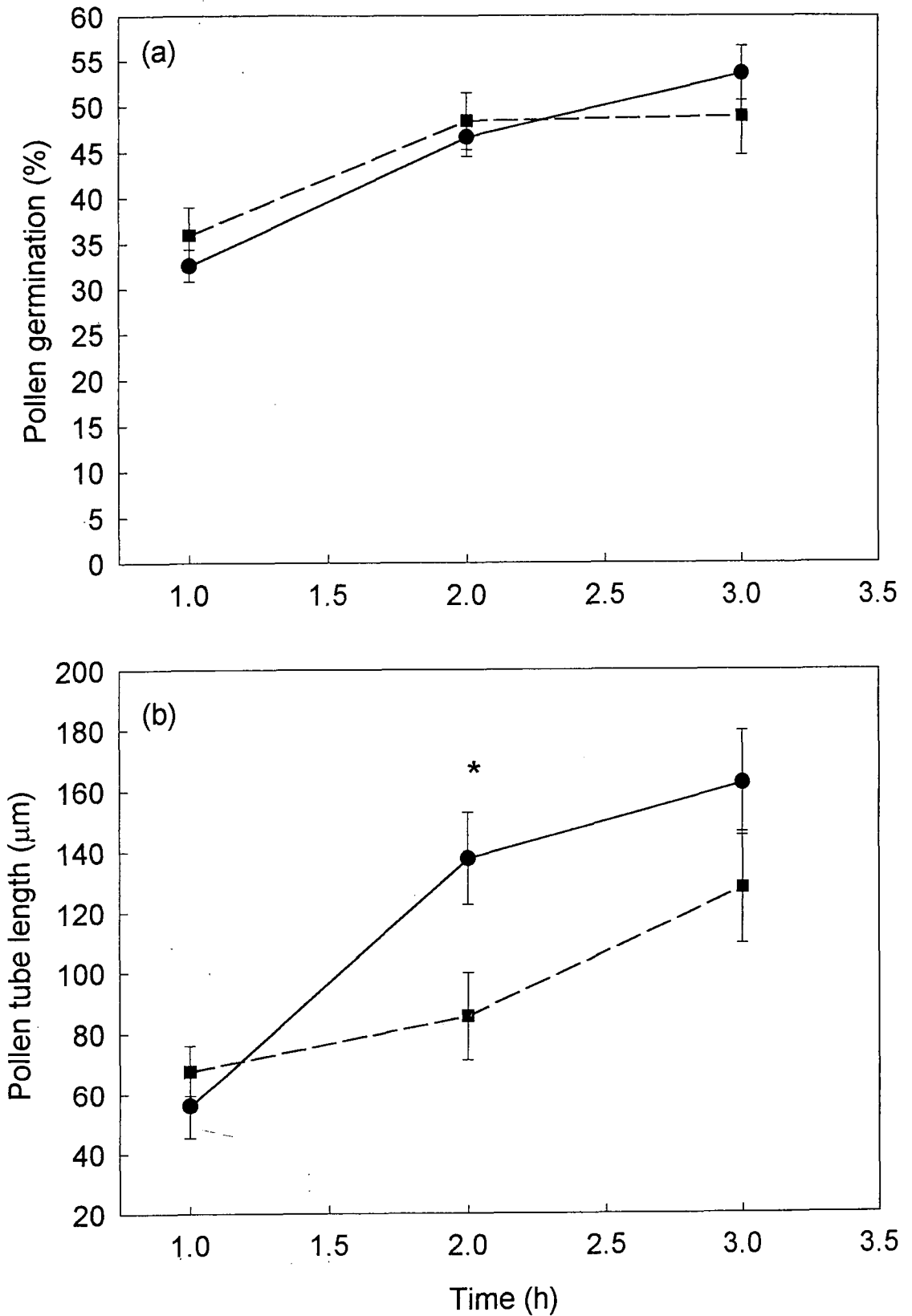


Figure 3.3. Percent pollen germination and pollen tube length of *Kalanchoe fedstchenkoi* in Roberts *et al.* germinating medium containing 15 % sucrose at two temperatures. ●, 20 °C; ■, 25 °C. Data represent the mean ± one SE of three replicates.

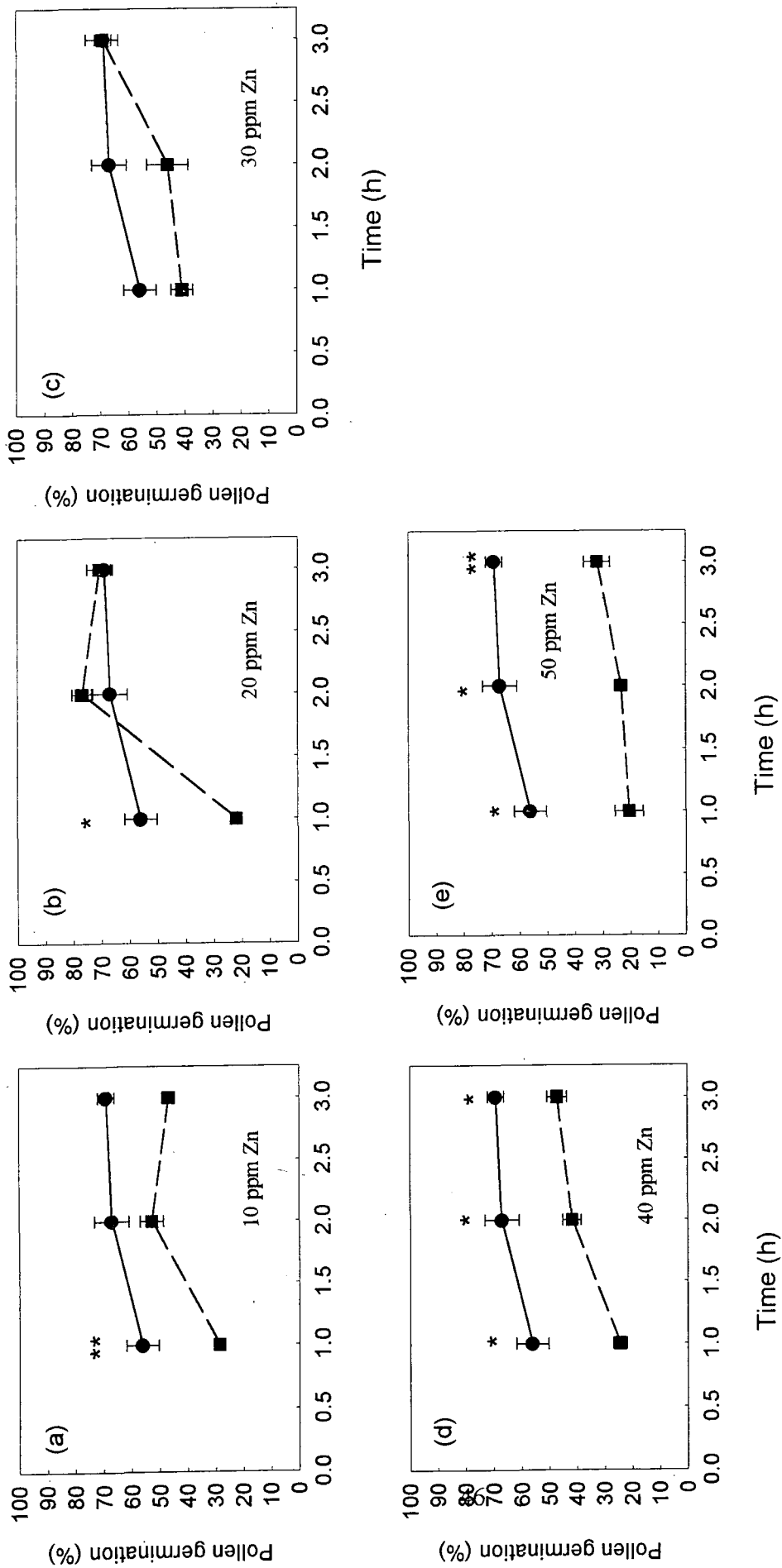


Figure 3.4. The effect of zinc upon percent pollen germination of *Kalanchoe fedtschenkoi* in Roberts *et al.* germinating medium containing either 0 (control; ●), 10, 20, 30, 40 or 50 ppm zinc (as ZnSO<sub>4</sub>; ■). Data represent the mean ± one SE of three replicates. \*, p ≤ 0.05; \*\*, p ≤ 0.01; \*\*\*, p ≤ 0.001 (Student's t-test). Full statistical analysis (ANOVA) is provided in Appendix 7.

reduced ( $p \leq 0.05$  to  $0.01$ ) and these differences were maintained over the 3 hour experimental period (reduction of 31.91 % and 53.81 % respectively).

A similar picture was also seen in the effect of Zn on PTL during the initial germination period (Fig. 3.5). Overall there were significant effects of Zn in the first hour of germination on PTL in all treatments. At 10 ppm, a significant effect of Zn on PTL ( $p \leq 0.01-0.05$ ) was observed over the 3 hour experimental period. However, at 20 ppm (Fig. 3.5 b), although there was a significant reduction of PTL at the first hour of germination, PTL seemed to later recover to the control value. This was shown by a non-significant difference to that of control. At 30 and 40 ppm Zn (Fig. 3.5 c, d), despite the initial significant reduction in PTL (at 1 hour) ( $p \leq 0.01$ ), the relationship between Zn and PTL was subsequently weaker. A relatively high variability between replicates at these later time points may, in part, account for this lack of significant difference between Zn and control treatments. At 50 ppm Zn, a much larger and sustained reduction in PTL (52.98 %) was observed over the entire 3 hour period.

### **3.2 Pollen germination and tube length of different *Brassica* genotypes**

There was no significant difference in %PG between these two genetic lines, *Brassica rapa self-compatible* and *dwf* (Fig. 3.6a). However, there was a significant difference in PTL (Fig. 3.6b), in particular, after 2 hours ( $p \leq 0.01$ ) in *dwf2*. The percent increase of the pollen tube length in genotype *dwf2*, compared with *self-compatible*, was 46 %.

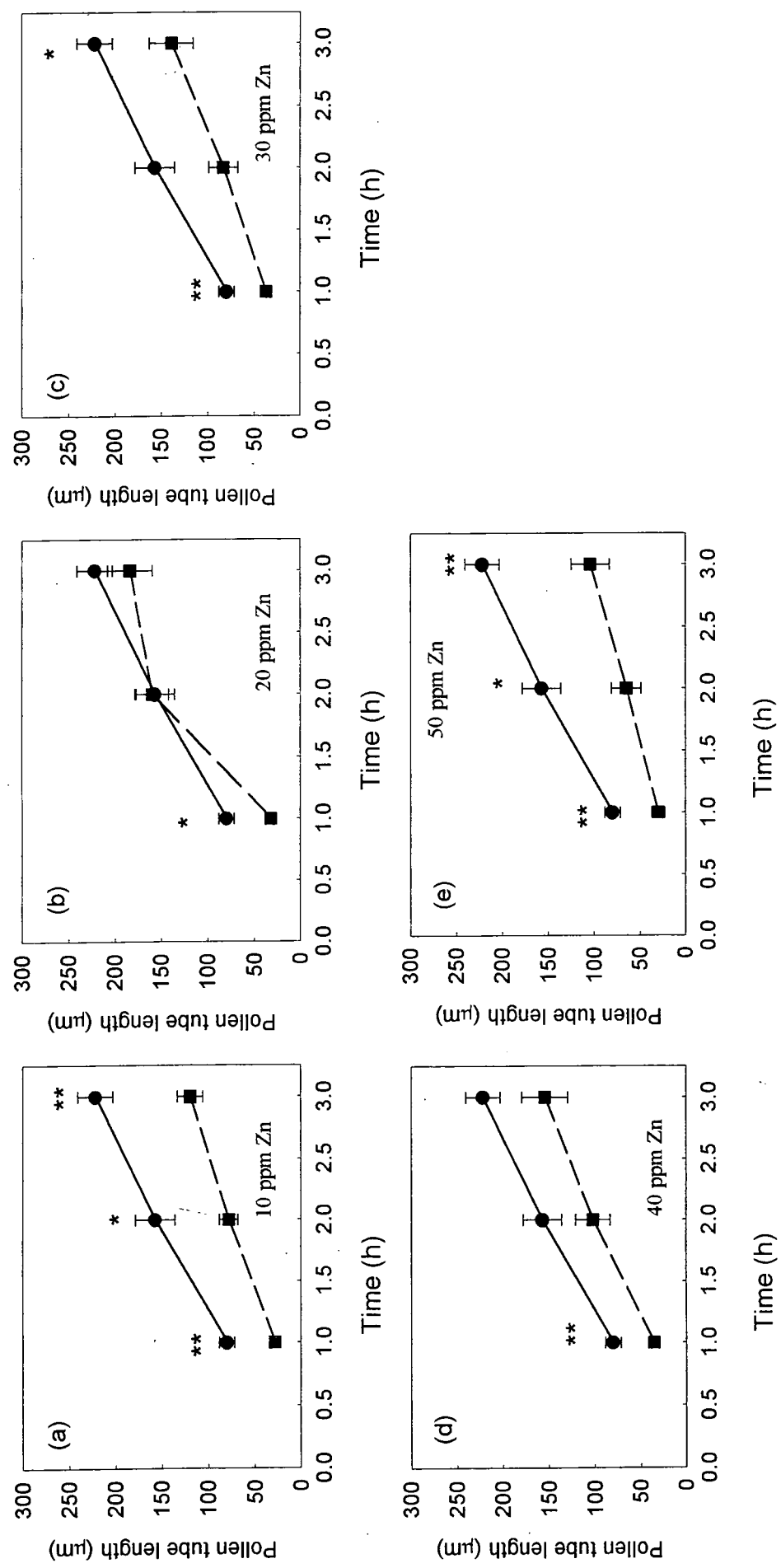


Figure 3.5. The effect of zinc upon pollen tube length of *Kalanchoe fedtschenkoi* in Roberts *et al.* germinating medium containing either 0 (control; ●), 10, 20, 30, 40 or 50 ppm zinc (as ZnSO<sub>4</sub>; ■). Data represent the mean ± one SE of three replicates. \*, p ≤ 0.05; \*\*, p ≤ 0.01; \*\*\* p ≤ 0.001 (Student's t-test). Full statistical analysis (ANOVA) is provided in Appendix 8.

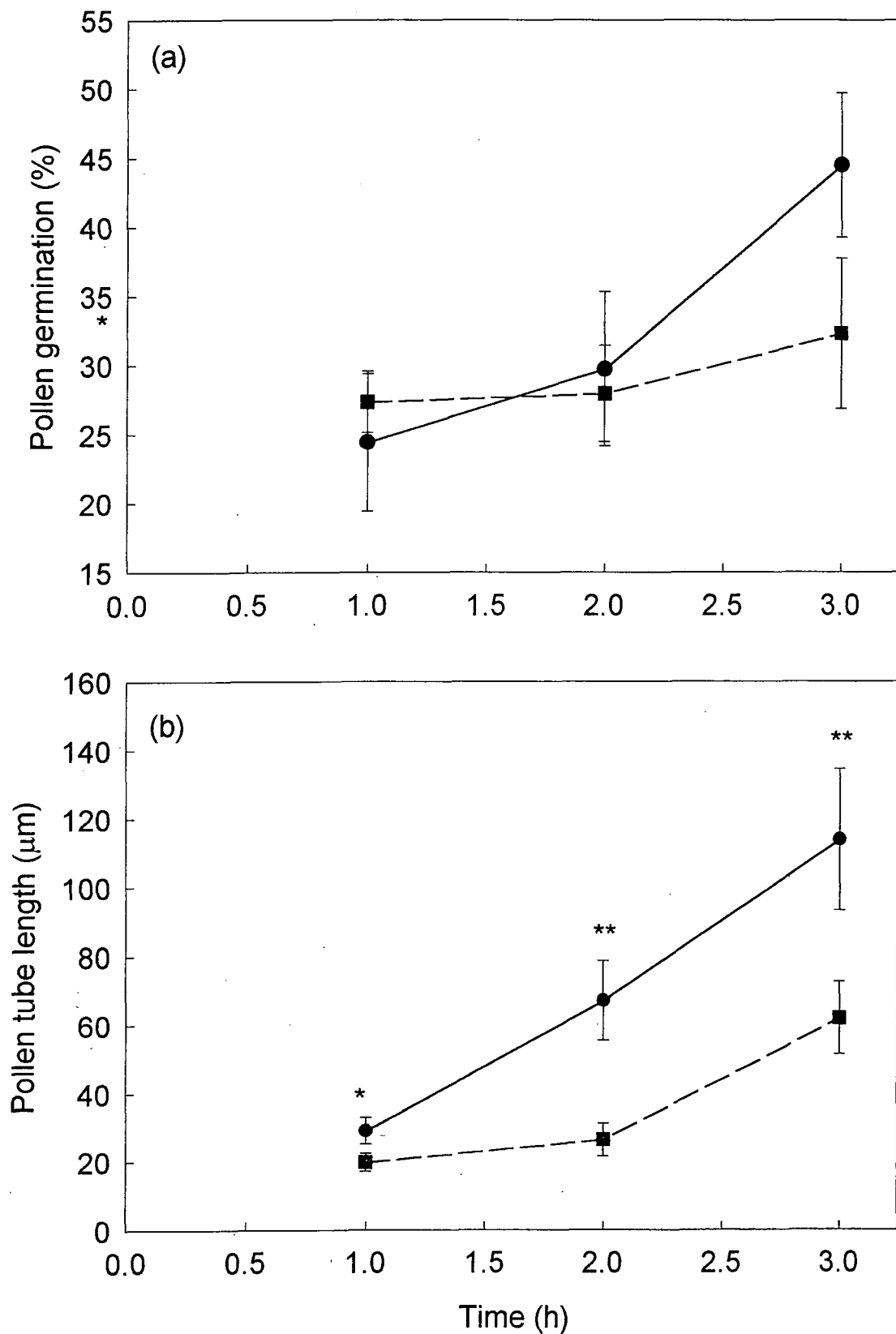


Figure 3.6. (a) Percent pollen germination and (b) pollen tube length of two genotypes of *Brassica rapa* in Robert's *et al.* germinating medium containing 15% sucrose at pH 8.0. ●, genotype *dwf2*; ■, genotype *Self*. Data represent the mean  $\pm$  one SE of three replicates. \*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*  $p \leq 0.001$  (Student's t-test). Full statistical analyses (ANOVA) are provided in Appendices 9 and 10.

### 3.3 Pollen viability of *Brassica rapa*, *dwf2*

Ten flowers were excised and their pollen viability was examined. There was no significant difference in mean percent of pollen viability between Flower 2 to Flower 10 (Fig. 3.7), which indicated good reproducibility in pollen viability. The average pollen viability of these 9 flowers was 37 %. However, there was a significant difference in Flower 1, which had a much lower per cent pollen viability. This might be due to age of flower.

### 3.4 Effect of different sucrose concentrations and PEG on pollen germination and pollen tube length *in vitro* of *Brassica rapa*, *dwf2*

Nine different media were prepared, containing different concentrations of sucrose and PEG in order to identify the best germination medium for pollen germination and tube growth of *Brassica rapa*, *dwf2*. Fig 3.8 a, c, and e shows that there were highly significant effects of sucrose concentrations, PEG concentrations and time at  $p \leq 0.001$  and all interaction of these treatments with time on %PG (see Appendix 12). The highest rate of germination was observed when using 15 % sucrose with the addition of 10 % PEG (Fig. 3.8 c). At higher concentrations of PEG, %PG was similar to treatment using sucrose alone. Therefore, the combination of 15 % sucrose and 10 % PEG was used as a suitable standard germinating medium for further experiments.

In 20 % and 10 % sucrose concentrations, %PG was lower than %PG in 15 % sucrose (Fig. 3.8 a, e), but again addition of 10 % PEG stimulated %PG. At a combination of 10 % sucrose plus 10 % PEG (Fig. 3.8 e), %PG increased over the 3 hour germinating period, though there was a slow germination rate over the first hour of the experimental period. In 20 % sucrose plus 10 % PEG (Fig. 3.8a), an increase in %PG was

observed in the initial germination period, but later the rate of %PG declined after 2 hours.

A similar pattern in the effect of PEG on PTL where high significant effect of sucrose concentrations, PEG concentrations and time on PTL at  $p \leq 0.001$  (Fig. 3.8 b, d, and f). The addition of 10 % PEG to 15 % sucrose had increased PTL, though this effect was not so clearcut, when comparing the 15 % PEG and 15 % sucrose alone treatments (Fig. 3.7 d). There were also stimulation effects of 10 % and 15 % PEG in the growth of PTL at 10 % sucrose over time (Fig. 3.8 f). An increased of PTL was also observed at 20 % sucrose and 10 % PEG (Fig. 3.8 b). There was, however, a negative interaction of PEG at 20 % sucrose and 15 % PEG which was probably due to the medium not providing the correct osmotic conditions for the germination of pollen.

### **3.5 Effect of heavy metals on pollen germination and tube length of *dwf2* in vitro**

To study the inhibitory effect of heavy metals on pollen germination and tube growth, two metallic salts, namely, zinc sulphate and copper sulphate were chosen. Three levels of zinc sulphate were added in the pollen germination medium: 20, 40, and 60 ppm. In separate experiments, three levels of copper sulphate were added in the pollen germination medium: 2, 4, and 6 ppm. Highly significant differences were observed between the metallic salt treatments and the control (with no metal addition).

#### **3.5.1 Effect of zinc sulphate**

There was a very highly significant effect of Zn on %PG ( $p \leq 0.001$ ) in all treatments (Fig. 3.9). However, the effect of Zn with time had a lower significance value (of  $p \leq 0.05$  (see Appendix 13)). There was a significant decrease in %PG in 20 ppm Zn

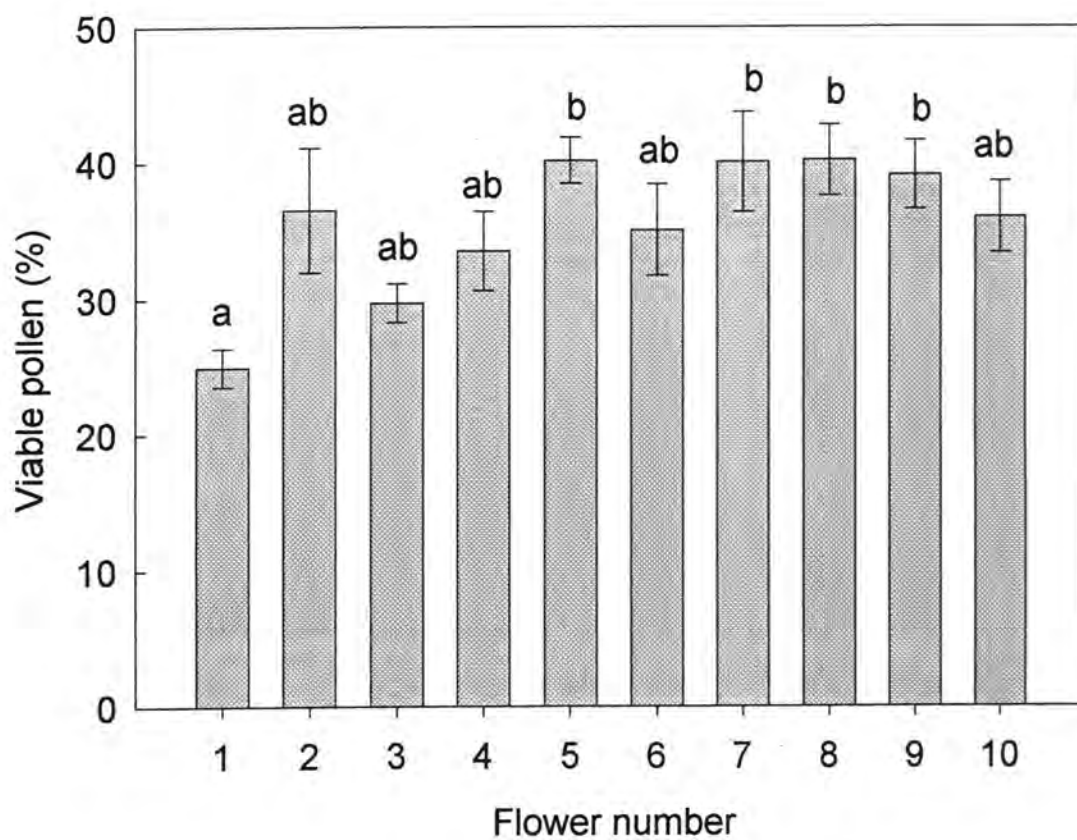


Figure 3.7. Mean percent pollen viability of ten counts per flower of *Brassica rapa dwf2*. Data represent the mean  $\pm$  one SE of ten separate determinations. Different letters above columns denote significant differences between means at  $p \leq 0.01$  critical level determined by Tukey's multiple comparison procedure (ANOVA). Data of proportions were arc-sin transformed prior to analysis.

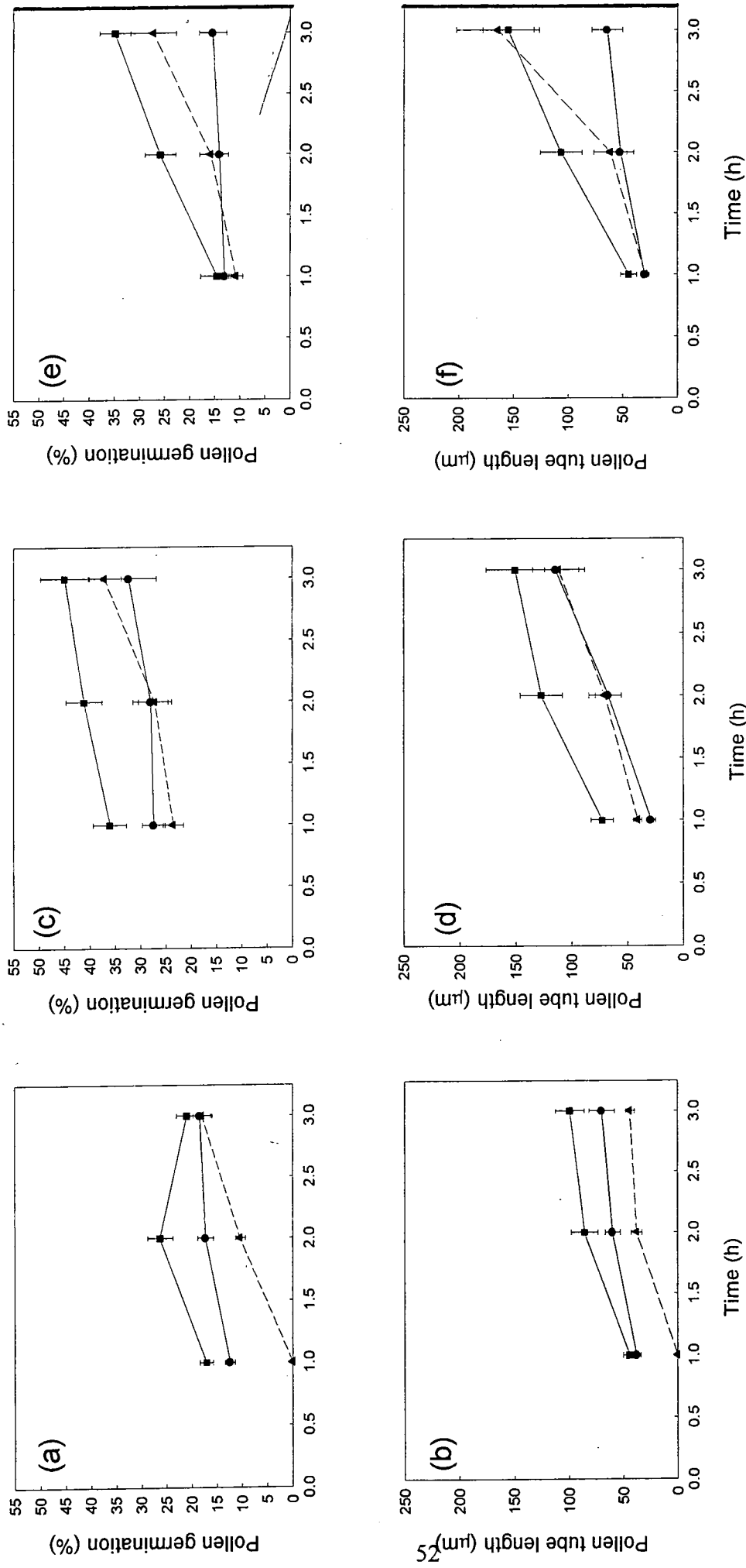


Figure 3.8. Percent pollen germination and pollen tube length of *Brassica rapa dwf2* in different sucrose (20 %, (a), (b); 15 %, (c), (d); 10 % (e), (f)) and PEG concentrations (●, 0 % PEG; ■, 10 % PEG; ▲, 15 % PEG). Data represent the mean ± one SE of 3 replicates.

for the first-two hours of the germination period (Fig. 3.9 a). After 3 hours, however, %PG returned to a value similar to that of the control (no metal addition). A similar pattern of response of %PG was observed at 40 ppm Zn (Fig. 3.9 c). In this instance, %PG was lowered over the entire 3 hour germination period; this effect was even greater at 60 ppm Zn (Fig. 3.9 e). Overall, there was a high significant effect of Zn concentration on %PG and this was shown by the extent of inhibition rising from 13 % at 20 ppm Zn to 65 % at 60 ppm Zn.

The effect of Zn on PTL was generally greater than on %PG. There was a very highly significant effect of Zn on PTL with time ( $p \leq 0.001$ ) (see Appendix 15). A significant reduction of PTL at 20 ppm Zn was observed over the 3 hour germinating period (Fig. 3.9 b). A similar pattern of response of PTL was observed at 40 ppm, but with a much more marked decrease in PTL ( $p \leq 0.001$ ) after 2 hour of the germination period (Fig. 3.9 d). A similar effect was also observed at 60 ppm (Fig. 3.9 f) in the first two hours of germination, but with a more significant reduction in PTL after 3 hours. Significant differences on PTL increased with increasing Zn concentrations. This was shown by the extent of the inhibition of PTL rising from 38 % at 20 ppm Zn to 69 % at 60 ppm Zn.

### **3.5.2 Effect of copper sulphate**

Unlike Zn, Cu seemed to have a large inhibitory effect on %PG at the first hour of germination period, even at low concentration of Cu (Fig. 3.10). There was a highly significant effect of Cu on %PG ( $p \leq 0.01$ ) at concentrations as low as 2 ppm (Fig. 3.10 a). As Cu concentrations increased to 4ppm Cu (Fig. 3.10 c), there was a further significant reduction of %PG ( $p \leq 0.01-0.001$ ), and even greater significant reductions at 6 ppm Cu over the 3 hour germinating period. The increasing concentrations of Cu

decreased the %PG. Its inhibitory impact on pollen germination ranged from 29 % at 2 ppm Cu to 52 % at 6 ppm.

Addition of Cu to the *in vitro* germination medium resulted in reduced PTL. Over the first-two hours of the germinating period, there was no significant effect of 2 or 4 ppm Cu on PTL (Fig. 3.10 b, d). The significant effect of Cu on PTL can only be observed at 3 hours from the start of the germinating period. However, at 6 ppm Cu, a significant reduction of PTL was observed at 2 hours. There was a delayed effect of Cu on the PTL, which can only be seen after 2-3 hours. This can be seen by rapid reductions in PTL after 2 hours in all treatments. The inhibitory impact of Cu on pollen tube length ranged from 33 % at 2 ppm Cu to 58 % at 6 ppm Cu.

### **3.6 *In vivo* pollen tube growth length of *Brassica rapa*, *dwf2***

In order to study the *in vivo* pollen tube growth length, six time intervals of 2, 4, 6, 8, 24, and 48 hours after pollination were selected. PTGL was observed to increase over the 2 to 6 hour period after pollination, but later the growth rate of pollen tube plateaued out 8 hours after pollination as shown in Fig. 3.11. The data indicated that after six hours of pollination, the pollen tube had reached its maximum length (552  $\mu\text{m}$ ). When observed under the microscope, the pollen tubes had reached the ovules (see Plate 3.1). The data also revealed that after 8 hours of pollination, maximum PTL was achieved.

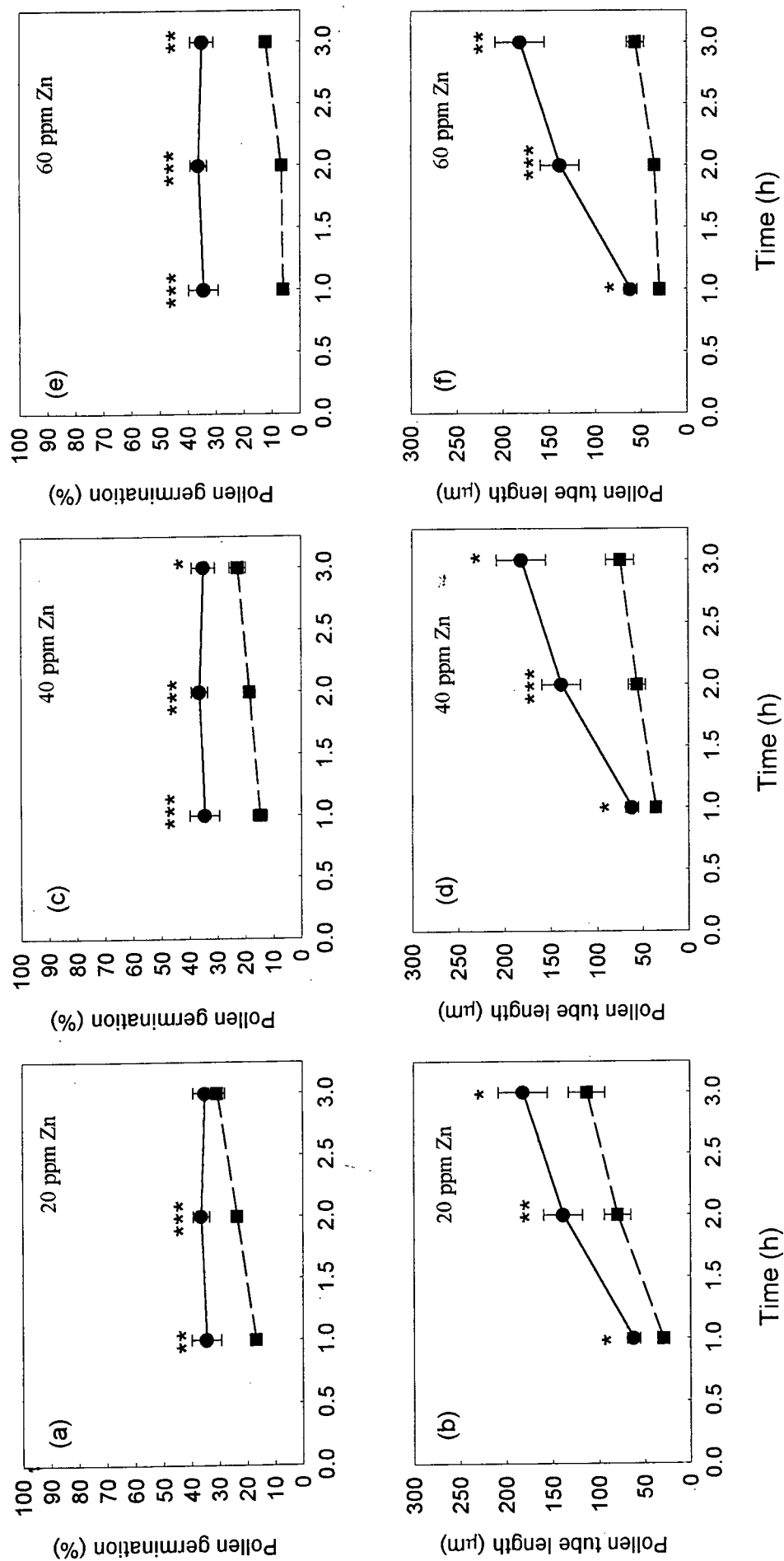


Figure 3.9. The effect of zinc upon percent pollen germination and pollen tube length of *Brassica rapa Dwf2* in Roberts *et al.* germinating medium containing 0 (control; ●), 10, 20, 30, 40 or 50 ppm zinc (as ZnSO<sub>4</sub>; ■). Data represent the mean ± one SE of three replicates. \*, p ≤ 0.05; \*\*, p ≤ 0.01; \*\*\*, p ≤ 0.001 (Student's t-test). Full statistical analyses (ANOVA) are provided in Appendices 14 and 15.

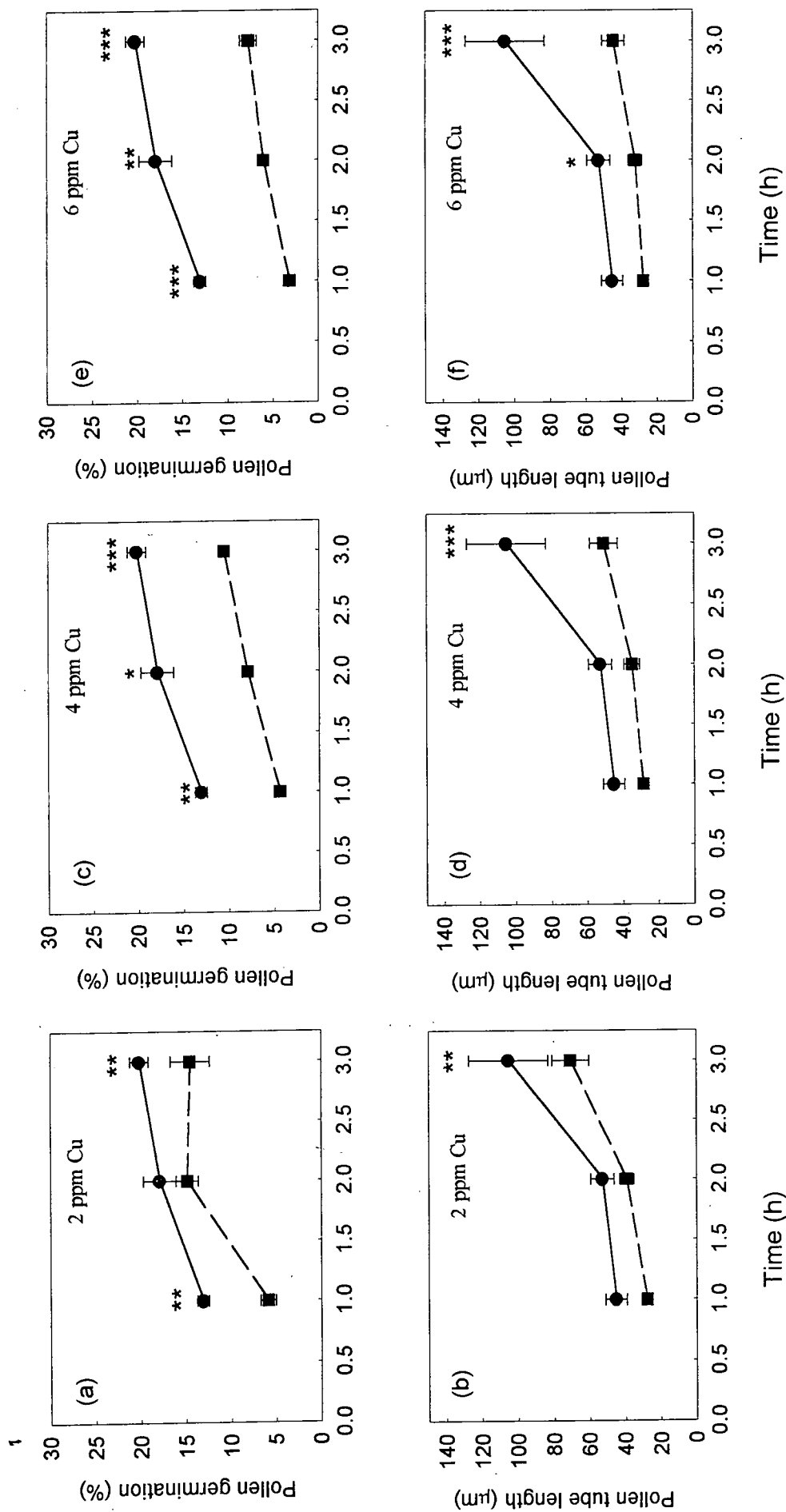


Figure 3.10. The effect of copper upon percent pollen germination and pollen tube length of *Brassica rapa Dwf2* in Roberts *et al.* germinating medium containing either 0 (control; ●), 2, 4 or 6 ppm copper (as  $\text{CuSO}_4$ ; ■). Data represent the mean  $\pm$  one SE of three replicates. \*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*  $p \leq 0.001$  (Student's t-test). Full statistical analyses (ANOVA) are provided in Appendices 16 and 17.

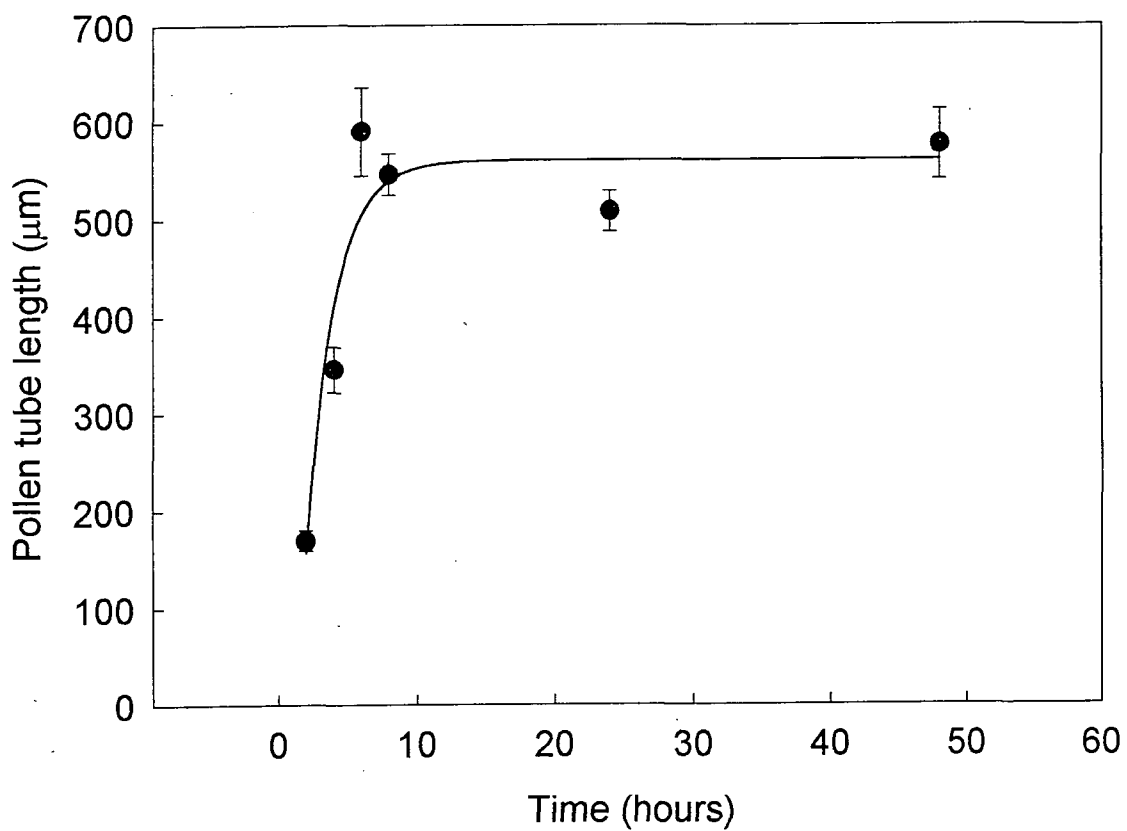


Figure 3.11. Pollen tube growth length *in vivo* of *Brassica rapa dwf2* at 2, 4, 6, 8, 24 and 48 hours after pollination. Data represent the mean  $\pm$  one SE of ten separate determinations. Fitted line is a modified hyperbola. Details of the fit can be found in Appendix 18.

### 3.7 Effect of heavy metals in pollen tube length growth *in vivo*

Several unforeseen problems were encountered, due to unsuitable growing conditions in the growth room, such as temperature control and the level of humidity at the time of experiments. Only plants grown in copper sulphate water culture medium produced flowers. Five flowers were hand-pollinated and left for six hours. Plants grown in a growth medium containing 2 ppm CuSO<sub>4</sub> showed a 97 % reduction in PTGL (Table 3.1;  $p \leq 0.001$ ). In addition, the toxicity of Zn and Cu on the development of the plants grown in the heavy metal water culture medium was observed. The plants showed retarded growth and chlorosis of the leaves (see Plate 3.2).

Table 3.1 Pollen tube growth length ( $\mu\text{m}$ ) *in vivo* in plants grown in CuSO<sub>4</sub> 6 hours after pollination

control (no added metal)	2 ppm CuSO <sub>4</sub>
590.76 $\pm$ 45.39	17.52 $\pm$ 3.13

### 3.8 The effect of heavy metals on pollen viability

Due to the very low numbers of flowers available and time limitations, only two flowers from plants grown in copper sulphate water culture were collected for analysis. Nevertheless, Fig. 3.12 shows that 2 ppm of copper sulphate solution caused a large significant reduction in the percentage of pollen viability of individual flowers, when compared to control flowers ( $p \leq 0.001$ ). The percentage reductions were 60 % and 34 % for Flower 1 and Flower 2 respectively.

### **3.9 Cytoplasmic streaming in pollen tubes of *Hippeastrum***

Two genetic lines of *Hippeastrum* were used in this study of cytoplasmic streaming. The rate of cytoplasmic streaming was found to generally increase with increasing pollen tube length (Fig. 3.13). In *cv. Appleblossom* (Fig. 3.13 b), the relationship between rate of cytoplasmic streaming and pollen tube length seemed to be linear, but the relationship was more complex in *cv. Belinda* (Fig. 3.13 a). To determine, more fully, the relationship between the rate of cytoplasmic streaming and pollen tube length in *cv. Belinda*, it would, with hindsight, be desirable to have had more data points, particularly at PTL's of between 320 and 450  $\mu\text{m}$ .

### **3.10 Effect of heavy metals in the cytoplasmic streaming of *Hippeastrum***

On addition of Zn to the germinating medium, the linear relationship between PTL and rate of cytoplasmic streaming appeared to hold (Fig. 3.14). The rate of cytoplasmic streaming decreased in addition of 50 ppm of Zn when compared to control over all the PTL points measured. However, similar to the control, the rate of cytoplasmic streaming increased with increasing PTL. At 100 ppm Zn, the slope of the line changed. At this concentration, the rate of cytoplasmic streaming declined as PTL increased.

### **3.11 The effect of heavy metals on pollen tube growth length of *Hippeastrum***

For both genetic lines of *Hippeastrum*, there was a highly significant effect of Zn on PTGL ( $p \leq 0.001$ ; Fig. 3.15). In 50 ppm Zn, *cv. Appleblossom* showed a 56.88 % reduction in PTL relative to the control (minus Zn). In comparison, *cv. Belinda* showed only a 22.78 % reduction in PTL at the same Zn concentration. Thus, *cv. Appleblossom*

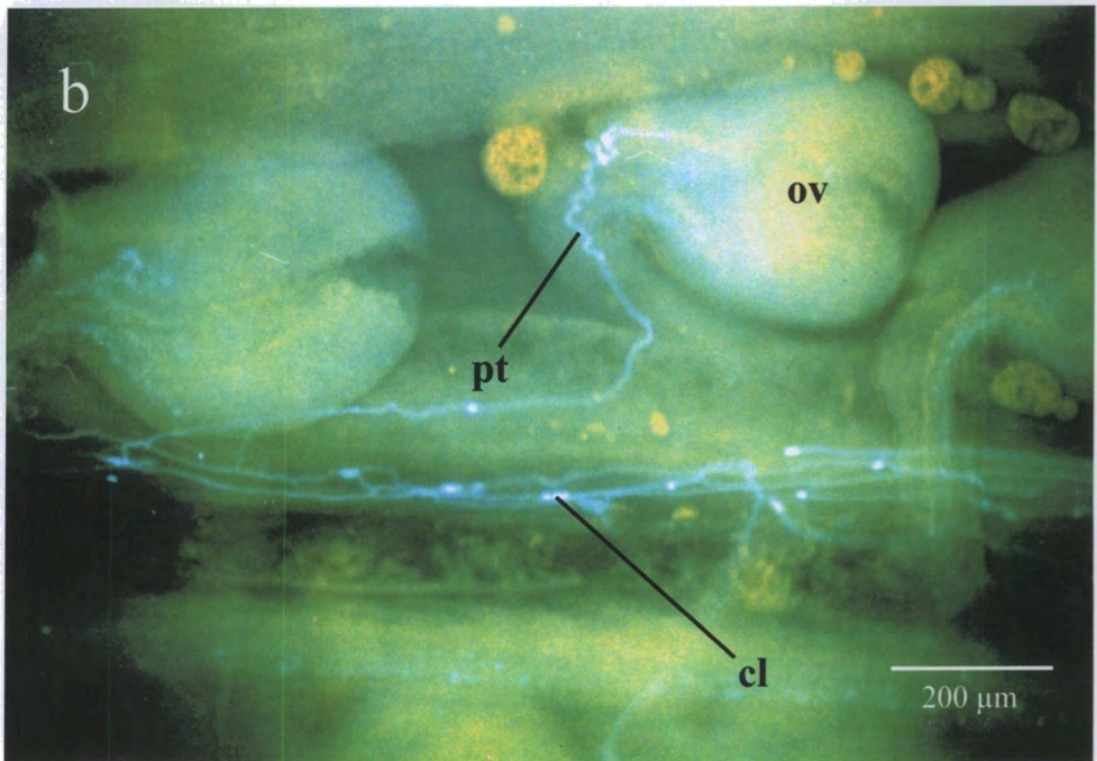
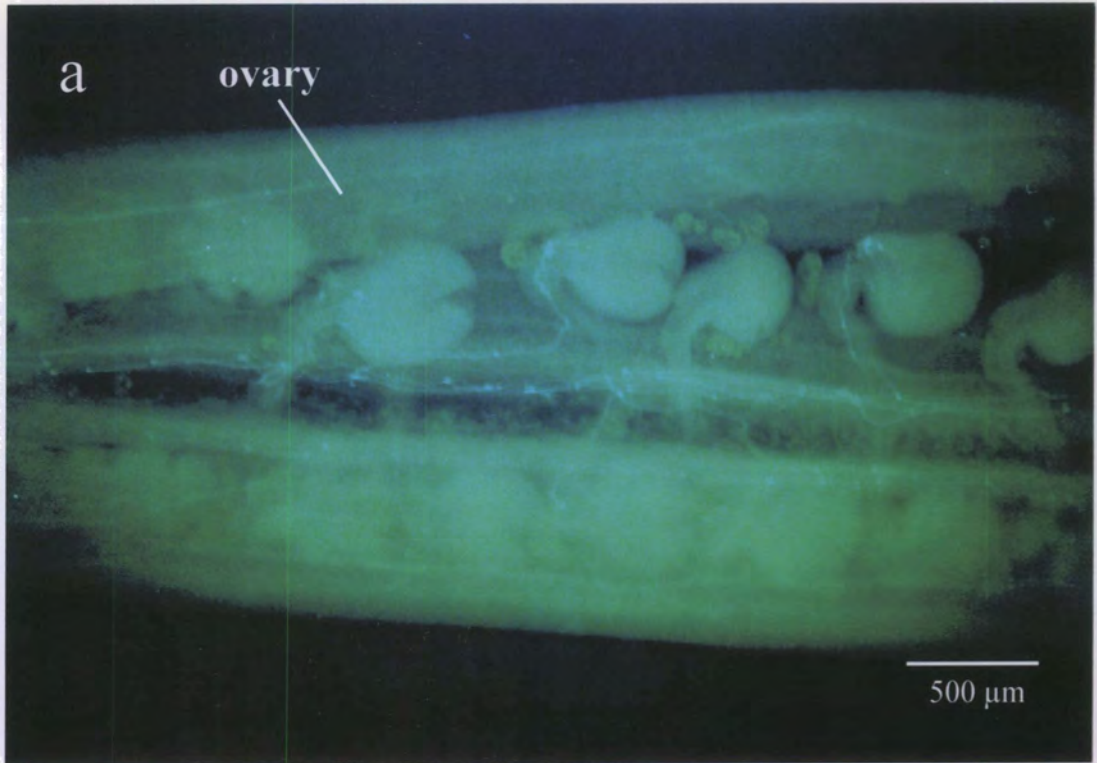


Plate 3.1. The entry of pollen tube into the ovule *in vivo*; (a) low magnification view, (b) high magnification view. cl: callose, ov:ovule, pt: pollen tube.

showed a much greater sensitivity to 50 ppm Zn than did *cv. Belinda*. At 100 ppm Zn, the reduction in PTL was similar in both genetic lines (67.79 % for *cv. Appleblossom* and 62.33 % *cv. Belinda*).

### **3.12 The effect of storage of pollen on pollen tube length of *Hippeastrum***

No significant differences were recorded in PTL *in vitro* in 20 % sucrose (control) due to length of storage between day 1 and day 3 (Fig. 3.16). By day 7, there was a significant reduction in PTL of 43 % ( $p \leq 0.05$ ). The effects of ZnSO<sub>4</sub> on PTL storage time period also showed no significant differences at 50 ppm ( $F = 223.15$ ,  $df = 2$ ,  $p = 0.042$ ) and at 100 ppm ( $F = 246.70$ ,  $df = 2$ ,  $p = 0.040$ ). PTL was found to decrease with increasing time of pollen storage prior to the *in vitro* germination test, and to decrease with increasing zinc concentration. Both the control and ZnSO<sub>4</sub> treatments had reduced PTL after the third and seventh day of storage (Fig. 3.16). The longer the duration of storage and the higher the concentration Zn, the greater the reduction of the PTL.



Plate 3.2. Plant grown in high concentration (40 p.p.m.) of  $\text{ZnSO}_4$  showing stunted growth and chlorotic leaves.

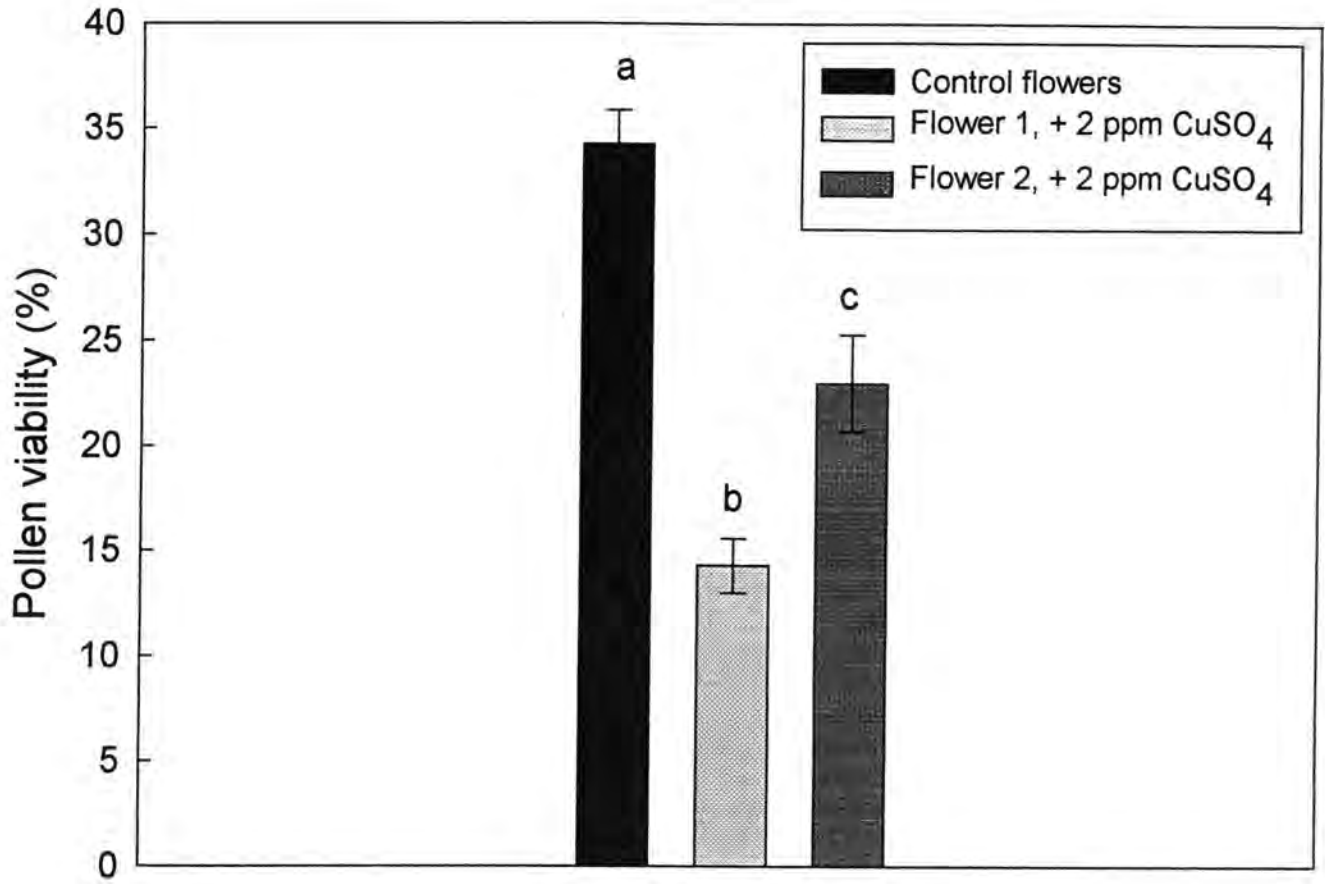


Figure 3.12. The effect of copper upon percent pollen viability in *Brassica rapa dwf2*. Data represent the mean  $\pm$  one SE of 7 separate determinations. Different letters above columns denote significant differences between means at the  $p \leq 0.05$  critical level determined by Tukey's multiple comparison procedure (ANOVA). Data for proportions were arc-sin transformed prior to analysis.

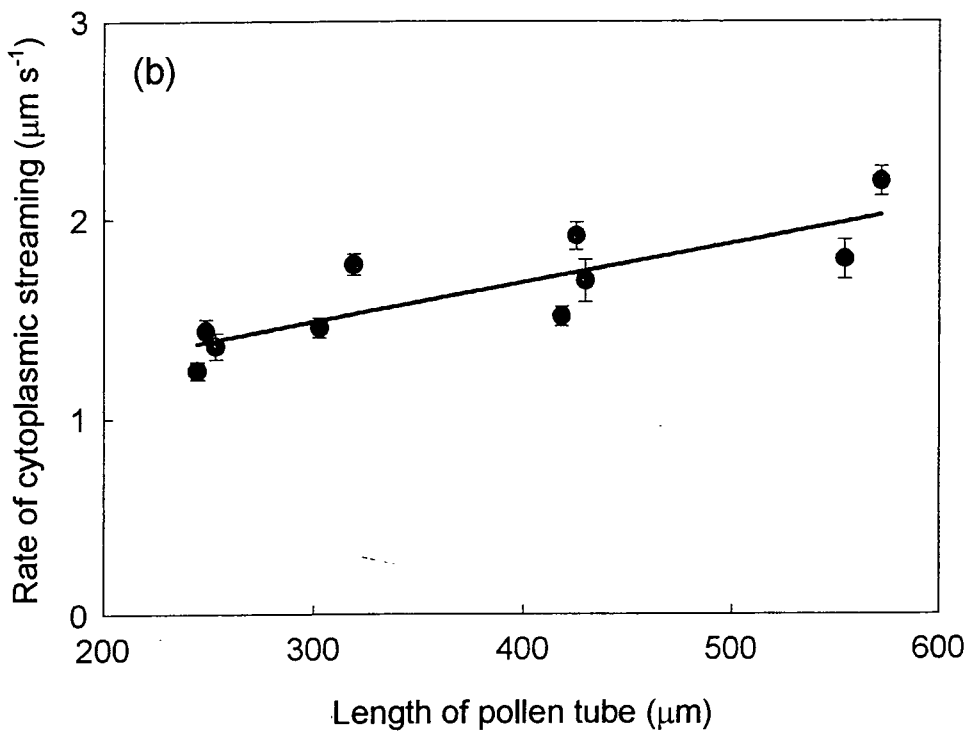
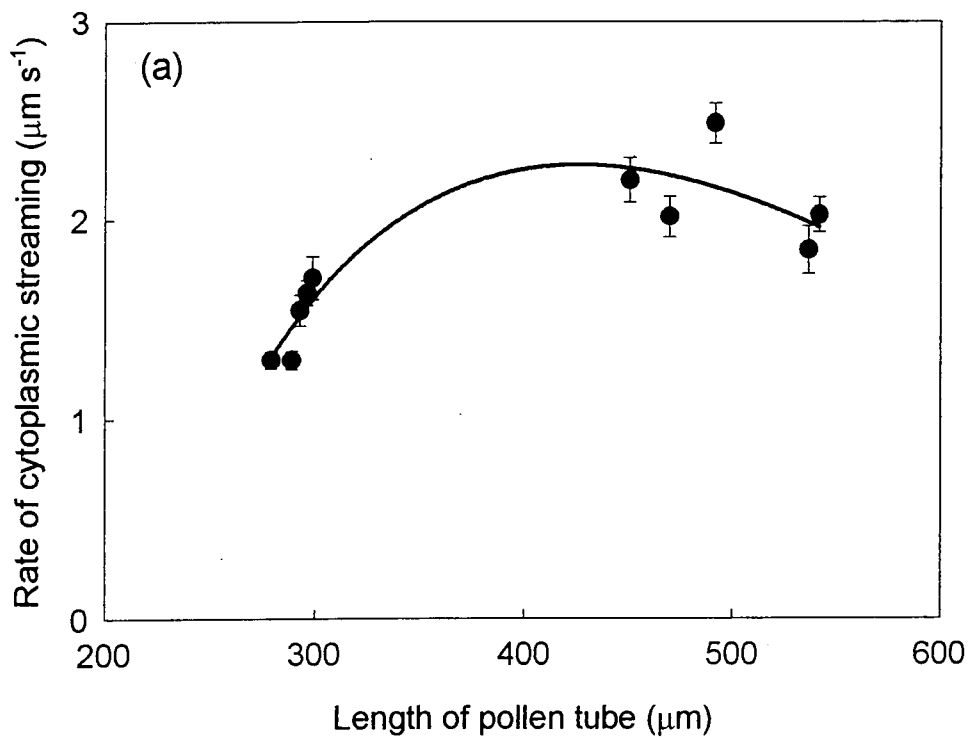


Figure 3.13. Movement of granules within the cytoplasm of pollen tubes of two genetic lines of *Hippeastrum* (a) cv Belinda and (b) cv Appleblossom three hours after incubation commenced at room temperature (*ca.* 20°C). Data represent the mean  $\pm$  one SE of 10 separate determinations. Full details of the fitted lines are given in Appendices 20 and 21.

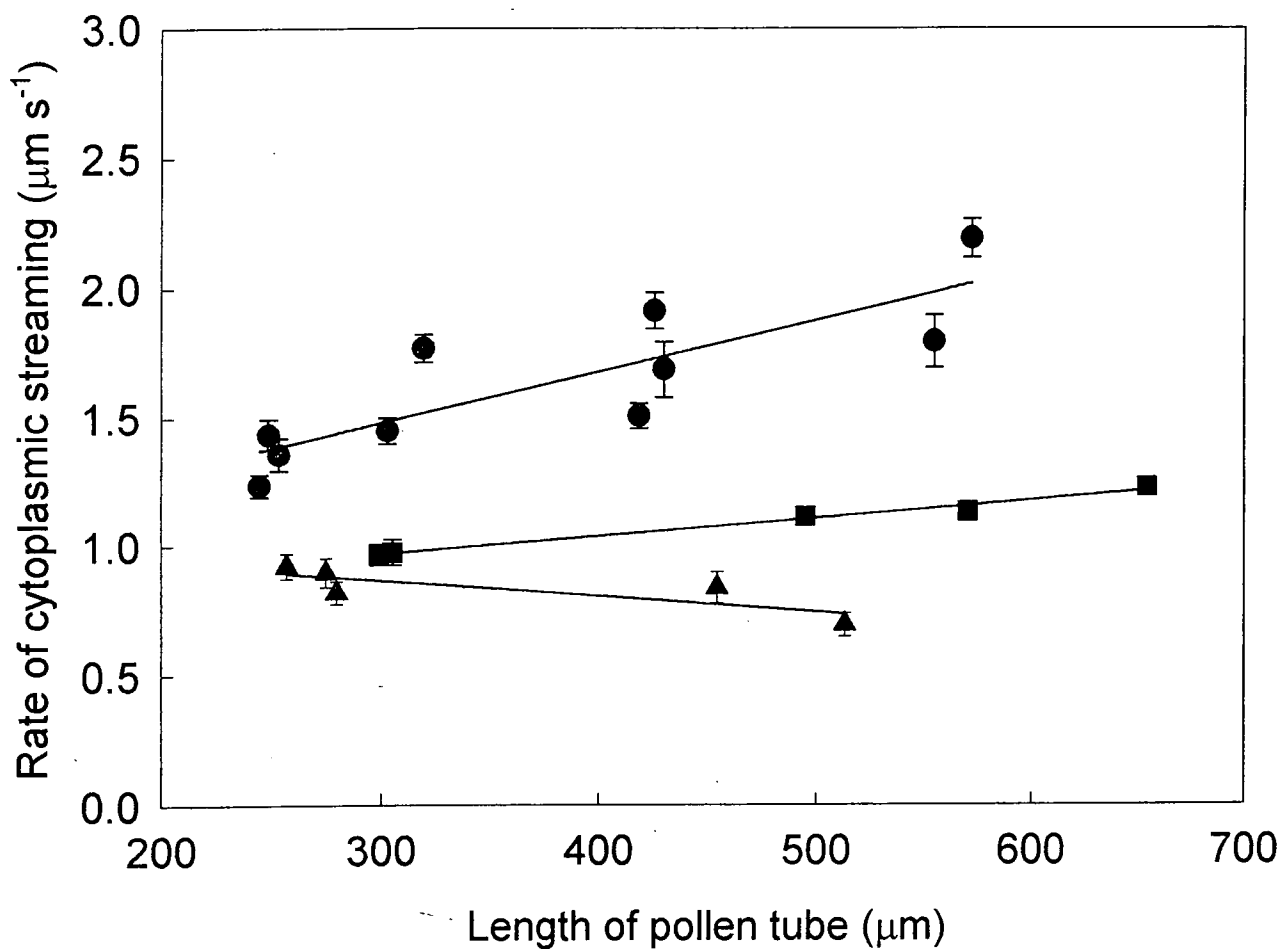


Figure 3.14. Movement of granules in pollen tubes of *Hippeastrum* cv. Appleblossom three hours after start of incubation. ●, control (0 ppm Zn); ■, 50 ppm Zn; ▲, 100 ppm Zn. Data represent the mean  $\pm$  one SE of ten separate determinations. Full statistical details of fitted lines are presented in Appendix 22.

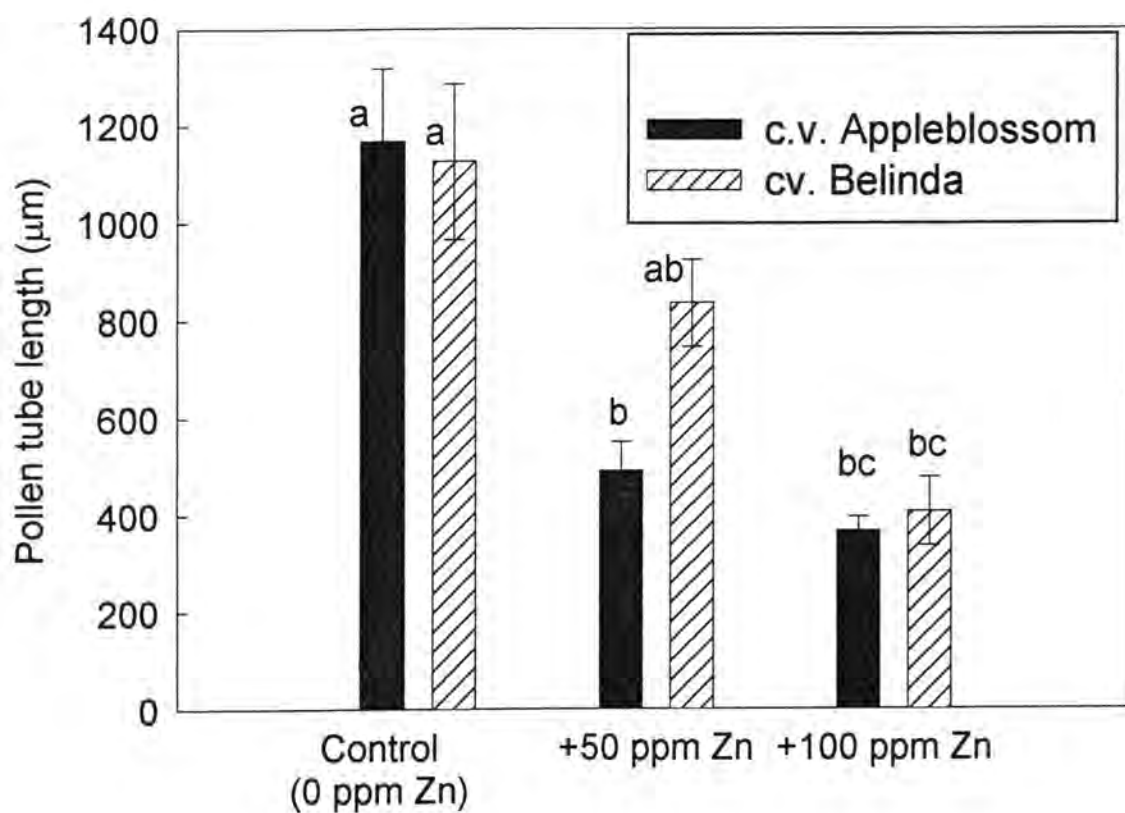


Figure 3.15. The effect of zinc (as  $ZnSO_4$ ) upon pollen tube growth in two genotypes of *Hippeastrum* (cv. Appleblossom, solid bars; cv. Belinda, hatched bars) after 5 hours incubation. Data represent the mean  $\pm$  one SE of 10 separate determinations. Different letters above columns denote significant differences between means at the  $p \leq 0.05$  critical level determined by Tukey's multiple comparison procedure (ANOVA).

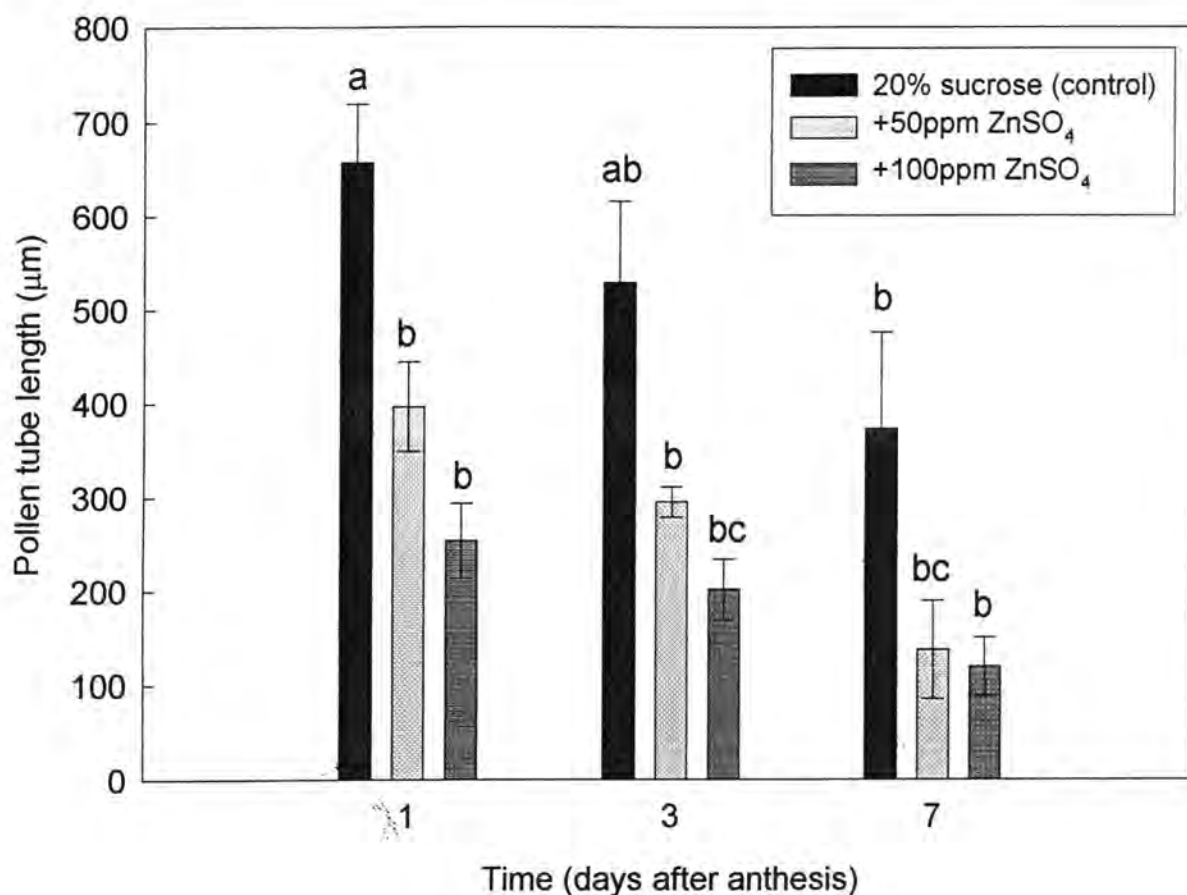


Figure 3.16. The effect of storage (age) on pollen tube growth length in *Hippeastrum* cv. Appleblossom three hours after incubation began. Data represent the mean  $\pm$  one SE of 10 separate determinations. Different letters above columns denote significant differences between means at the  $p \leq 0.05$  critical level determined by Tukey's multiple comparison procedure (ANOVA).

## Chapter IV

### DISCUSSION

#### 4.1 *In vitro* Germination media

A number of studies have shown that although there are general requirements for certain media components for pollen germination *in vitro*, and that there are many species that have specific germination requirements. A basal medium of sucrose (to provide the correct osmotic conditions and an energy source), boron and calcium salts is the usual starting point for investigating species-specific germination media requirements.

In a simple germination medium containing sucrose solution, 50 ppm boric acid and 2000 ppm calcium nitrate, *Kalanchoe fedtschenkoi* had achieved low %PG and PTL, but satisfactory %PG and PTL were achieved in Roberts *et al.* (1983) medium containing sucrose solution, boric acid, calcium chloride, potassium nitrate and Tris. Apart from the common requirements for pollen germination: sucrose, boric acid and calcium, *Kalanchoe fedtschenkoi* benefits from Tris to enhance germination of pollen and rate of tube growth. However, with the addition of 10 % PEG, there were variations in %PG and PTL in different concentrations of sucrose. There was no significant improvement in percentage germination of pollen in the addition of PEG in all treatments over the 3 hour germinating period. Nevertheless, addition of PEG had increased PTL in 10 % sucrose.

Under the same nutritional requirements with 15 % sucrose of Roberts *et al.* (1983) germinating medium, two genetically distinct lines of *Brassica rapa*, *Self-compatible* and *dwf2* (Fig. 3.6) were tested. There was no difference in percent pollen germination between *Self-compatible* and *dwf2*, however *dwf2* did exhibit increased pollen tube length. This result indicated that both genotypes responded differently in terms of pollen tube growth.

In further experiments with *Brassica rapa dwf2*, PEG was added to Robert's *et al.* (1983) germinating medium. The addition of PEG improved the percentage pollen germination and pollen tube length in 20 % sucrose, 15 % sucrose and 10 % sucrose. The best response was at low concentrations of sucrose (15 % and 10 %). This was in accordance with the results of Shivanna and Sawhney (1995) who have achieved high percentage pollen germination and good pollen tube growth by using low sucrose concentrations. In two different concentrations of PEG (10 % and 15 %) with 20, 15 and 10 % sucrose, the maximum pollen tube growth in *Brassica rapa dwf2* was obtained in media with 15 % PEG and 10 % sucrose after 3 hours. However, the best response in percent pollen germination and pollen tube growth in *Brassica rapa dwf2* was obtained in the medium with 10 % PEG and 15 % sucrose after 3 hours.

There have been studies on the harmful effects of temperature regimes on plant species which may affect pollen germination and pollen tube growth. While low temperatures may result in pollen abortion (Younger, 1961), high temperatures can cause bursting of pollen (Johri and Vasil, 1961), which consequently reduces pollen germination and tube length. The suitable temperature for optimum pollen germination under normal conditions is between 20 and 30 °C (Johri and Vasil, 1961) and temperatures between this range were used in this study in *Kalanchoe fedtschenkoi* (20 °C and 25 °C) (Fig. 3.3). It was found that there was no difference in %PG, however, there was a significant increase in PTL at 20 °C which was in accordance to the findings of Brink (1924a, b, c), that pollen tube growth is sensitive to temperature.

Although similar germination media were used for the plant species under study in the present investigation, it was found that the optimum requirements for the plants in question were species-specific. From this study, it was found that the best *in vitro* germination media for the maximum pollen tube growth in *Brassica rapa dwf2* were media with 15 % PEG and 10 % sucrose when monitored after 3 hours at 20°C; while in *Kalanchoe fedtschenkoi*, 10 % PEG and 10 % sucrose gave the maximum %PG and PTL.

#### 4.2 *In vivo* pollen tube growth length of *Brassica rapa dwf2*

The results from Figure 3.11 revealed that the pollen tube of *Brassica rapa dwf2* pollen reached its maximum growth length of 552  $\mu\text{m}$  six hours after pollination. The growth rate of the pollen tube was 92  $\mu\text{m}/\text{h}$ , however no comparable *in vitro* data was taken. Two hours after pollination, the pollen tube growth rate was 28  $\mu\text{m}/\text{h}$ , while *in vitro* pollen tube growth over 2 hours was 11  $\mu\text{m}/\text{h}$ . The results found in this study supported the idea that the pistil tissue provides much more suitable growth conditions for pollen tube than that provided *in vitro*. A great reduction of 97 % in pollen tube growth was recorded when 2 ppm  $\text{CuSO}_4$  was added to the plant growth medium. This would suggest that plants which were grown in heavy metals had declined pollen tube growth rates of pollen which would reduced the male success relative to plants grown in better conditions. Since pollen tubes are vital for the plant reproductive system, this could play a decisive role for the whole plant (Sawadis and Reiss, 1995).

#### 4.3 The effect of heavy metals on percent pollen germination and pollen tube length

A few pollen researchers have investigated the effects of heavy metals on certain heavy metal-sensitive and -tolerant pollen types. Remarkable losses in pollen germination and reductions in pollen tube growth have been reported. Brewbaker and Kwack (1964) reported an inhibitory effect of zinc at 100  $\text{mg l}^{-1}$  ( $\equiv$  100  $\mu\text{g ml}^{-1}$  or 100 ppm) and copper at 30  $\text{mg l}^{-1}$ ; Bhandal and Bala, (1989) recorded a reduction of 52 % pollen germination when they treated *Amaryllis vittata Ait* (Amaryllidaceae) in 200  $\mu\text{M}$   $\text{Zn}(\text{NO}_3)_2$  and 77 % pollen tube growth inhibition after 2 hours of the experiment, and a complete inhibitory effect of copper was observed at 150  $\mu\text{M}$ .

In the present study, the toxic effect of Zn on percent pollen germination and pollen tube length of *Kalanchoe fedtschenkoi* was observed at 50 ppm Zn, with similar percentage reduction of pollen germination and pollen tube length (53.81 % and 52.98 % respectively) over the three hour germinating period. The greatest effect of toxicity of Zn on the percent pollen germination and pollen tube length of *Brassica rapa dwf2* was at a high concentration of Zn (60 ppm), and occurred at the early phase of the germinating period (1-2 hours), but subsequently weakened at 3 hours. It seemed that pollen could become tolerant to Zn over time. It could have been a specific phase of pollen tube growth that was more sensitive to Zn than later phases.

In two genetically distinct lines of *Hippeastrum*, cv. *Appleblossom* was more sensitive to Zn than cv. *Belinda* at 50 ppm. However, both genetic lines had showed a similar response in 100 ppm Zn. This might indicate that pollen tube growth rate is influenced by the gametophytic genotype at low concentrations, but that this factor is less important at high concentrations.

Previous studies have shown that  $\text{Cu}^{2+}$  was found to be more toxic than Zn on percent pollen germination and pollen tube growth. This was again shown in this study by the large inhibitory effect of Cu on the percentage pollen germination of *Brassica rapa dwf2* in the early and later stages of germination, at much lower concentrations than Zn. However, the effect in the reduction of pollen tube length was only observed at 3 hours. This delayed effect of Cu on pollen tube growth might show that Cu might affect the second phase of *in vitro* growth curves (rapid elongation period or the linear growth phase), which otherwise pollen tubes could grow at a linear rate under optimal conditions (Brewbaker and Majumder, 1961). The percentage reductions were 52 % and 58 % for pollen germination and tube length respectively.

#### 4.4 Pollen viability of *Brassica rapa*, *dwf2*

The percent viability of *Brassica rapa*, *dwf2*, measured by the fluorescein test (FCR), was 37 % and the percent *in vitro* pollen germination of the same genotype was 32 %. Naturally, some of the pollen grains become sterile and since FCR measures the integrity of the plasmalemma, rather than germination itself, it may overestimate viability in some cases (Shivanna and Heslop-Harrison, 1981). Shivanna *et al.* (1991) also stated that the FCR test reflects the ability of the pollen to germinate, but does not reflect the vigour of the pollen. In their studies on the relationship between *in vitro* pollen germination and FCR, Shivanna and Heslop-Harrison, (1981) found that FCR correlates well with *in vitro* germination in a range of species. Before studies on pollen viability were undertaken, environmental differences in pollen viability would be reduced by carefully controlling the freshness of pollen used in hand-pollination.

In the present investigation, there was a very marked reduction in percentage of pollen viability by Cu as shown in Table 3.1. This loss of pollen viability was noted in previous studies by Handique and Bala (1995) and by Bhandal and Bala (1989), who attributed the loss of pollen viability to heavy metals ions penetrating the pollen grains, resulting in inactivation and interfering with the functioning of enzymes leading to loss of pollen viability.

#### 4.5 Cytoplasmic streaming in pollen tubes of *Hippeastrum*

The results from Figure 3.14 showed a gradual increase in the rate of cytoplasmic streaming *in vitro* in pollen tubes of *Hippeastrum* cv. *Appleblossom*, as the length of the pollen tubes increased. After addition of 50 ppm Zn, the rate of cytoplasmic streaming had decreased. Further addition of Zn to a final concentration of 100 ppm Zn resulted in a

further decrease in the rate of cytoplasmic streaming, while pollen tube length continued to increase. This might indicate that the rate of cytoplasmic streaming could be an indicator of heavy metal toxicity. It is thought that the metal applied to the pollen culture was almost all bound to the cell wall, and only small amounts, if any, were taken up into the cytosol since there was no distinct effect observed in pollen tubes. If some metal accumulated intracellularly, the time until the tubes stop growth and burst may have been too short to allow manifestation of visible intracellular effects (Sawadis and Reiss, 1995). When this happened, it could affect the rate of cytoplasmic streaming by slowing down the streaming movement. Thus the rate of cytoplasmic streaming *in vitro* is a useful assay for detecting the toxicity of heavy metals.

However, there are several problems that may be encountered when using this kind of assay. These include: the difficulty of keeping track of the chosen granule along its journey within a specified distance, due to its unstable movement within the pollen tube; the duration of observation should be short as possible since if a sample was kept too long for observation under the microscope, the pollen under the microscope cover slip may be deprived of a supply of oxygen, thus may stop the cytoplasm from streaming.

#### **4.6 The effect of storage of pollen on pollen tube length of *Hippeastrum***

Stored pollen usually germinated slowly and required a longer time period to reach maximum germination than did fresh pollen. In this study, pollen stored at room temperature (at *ca.* 20 °C) for seven days, gave rise to a shorter pollen tube length in *cv. Appleblossom* in all treatments, including the control (0 ppm Zn). A similar finding was recorded by Jain and Shivanna (1990) who stored pollen grains in hexane under laboratory conditions and found that pollen tube length of *Crotalaria retusa* was very much reduced in stored pollen after 30 days. These authors stated that loss of vigour seems to be a general response of stressed pollen due to storage. This might be due to utilisation of

stored food materials for chemical processes like respiration, desiccation and inactivation of enzymes, as suggested by Johri and Vasil (1961). Thus, there would be an argument as to whether pollen used must be fresh or stored. When using fresh pollen, the continuous supply of fresh pollen could only be obtained from test plants that produce flowers all the time. In this present study, *Brassica rapa* is a rapid-cycling plant that produces abundant flowers all the year round. However, for *Hippeastrum* which produces flowers only during spring season, the pollen needs to be stored in a suitable storage condition such as in liquid nitrogen, to preserve the viability and fertility of pollen for a long period.

In the present study, the addition of Zn had the greatest effect on the pollen tube length. This indicated that as the pollen aged, its sensitivity to heavy metals may have increased. This could possibly be explained by the fact that large concentrations of heavy metals may interfere with the pectin-calcium binding sites and cause a decrease in cell wall elasticity (Matsumoto *et al.*, 1977). Consequently, normal pollen tube growth will be inhibited, the cell diameter will increase, and cell walls become thicker. Work by Roderer and Reiss (1988) found that inorganic lead (regardless of the salts used) affects pollen tube growth by interfering with cell wall synthesis. This was shown by Sawadis and Reiss (1995) by observing the effect of heavy metals on ultrastructure of pollen tube and who found that at the electron microscope level, the most distinct effect of heavy metal treatment was the abnormally organised cell wall at or near the tip. Therefore, this would affect the growth of pollen tube length.

The present study has aimed to develop an *in vitro* heavy metal assay based upon the sensitive male gametophyte (pollen) of flowering plants. It has shown that it is essential to have a standardised germination medium plus a standardised study species and growth conditions. In addition, the test environment and techniques employed must clearly be reproducible and standard in their execution. Finally, the period of experimentation, in terms of pollen tube growth extension, must also be standard in length since effects of heavy metal upon germination and subsequent pollen tube growth may be dependent upon the period of exposure to the heavy metal insult. This study goes some way to identify important variables that must be controlled if such an assay is to be

successful and reproducible. However, following this initial study, further work is required before such a toxicity assay could be used reliably and routinely. There are still a number of questions regarding uptake of heavy metals from soil growth media into the male gametophyte. Such challenging issues will remain important aspects of any further work and application of findings into the general field environment in instances where heavy metal pollution of ground materials remains a significant issue throughout many parts of the industrialised world.

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## Appendices

### Appendix 1

Two-Way Analysis of Variance for Percent pollen germination of *Kalanchoe fedtschenkoi* in different sucrose concentrations in simple germination medium. (refer to Fig. 3.1 (a), \*\*\*  $p \leq 0.001$ ; \*\*  $p \leq 0.01$ ; \*  $p \leq 0.05$ ; NS, not significant).

Source	DF	SS	MS	F	P	
Treatment	2	543.79	271.90	6.57	0.004	***
Time	2	1119.24	559.62	13.52	0.000	***
Treatment /Time	4	526.08	131.52	3.18	0.025	*
Error	36	1490.22	41.40			
Total	44	3679.33				

### Appendix 2

Two-Way Analysis of Variance for Pollen tube length of *Kalanchoe fedtschenkoi* in different sucrose concentrations in simple germination medium. (refer to Fig. 3.1 (b), \*\*\*  $p \leq 0.001$ ; \*\*  $p \leq 0.01$ ; \*  $p \leq 0.05$ ; NS, not significant).

Source	DF	SS	MS	F	P	
Treatment	2	2352.1	1176.0	5.90	0.006	*
Time	2	495.2	247.6	1.24	0.301	NS
Treatment /Time	4	1229.6	307.4	1.54	0.211	NS
Error	36	7176.3	41.40			
Total	44	11253.1				

### Appendix 3

Multiple-Way Analysis of Variance for Pollen germination of *Kalanchoe fedtschenkoi* in Robert's *et al.* germinating medium containing different concentrations of sucrose and PEG at pH 6.0 (refer to Fig. 3.2 (a, c, e), \*\*\*  $p \leq 0.001$ ; \*\*  $p \leq 0.01$ ; \*  $p \leq 0.05$ ; NS, not significant).

Source	DF	SS	MS	F	P	
PEG conc	1	232.30	232.30	5.01	0.028	*
Sucrose	2	1861.68	930.84	20.09	0.000	***
Time	2	3730.25	1865.13	40.25	0.000	***
PEG conc/Sucrose	2	825.73	412.87	8.91	0.000	***
PEG conc/Time	2	25.20	12.60	0.27	0.763	NS
Sucrose/Time	4	1029.49	257.37	5.55	0.001	**
PEG conc/Sucrose/Time	4	1220.22	305.05	6.58	0.000	***
Error	72	3336.68	46.34			
Total	89	12261.54				

### Appendix 4

Multiple-Way Analysis of Variance for Pollen tube length of *Kalanchoe fedtschenkoi* in Robert's *et al.* germinating medium containing different concentrations of sucrose and PEG at pH 6.0 (refer to Fig. 3.2 (b, d, f), \*\*\*  $p \leq 0.001$ ; \*\*  $p \leq 0.01$ ; \*  $p \leq 0.05$ ; NS, not significant).

Source	DF	SS	MS	F	P	
PEG conc	1	61007	61007	98.18	0.000	***
Sucrose	2	65019	32510	52.32	0.000	***
Time	2	289137	144568	232.66	0.000	***
PEG conc/Sucrose	2	25984	12992	20.91	0.000	***
PEG conc/Time	2	12236	6118	9.85	0.000	***
Sucrose/Time	4	5373	1343	2.16	0.082	NS
PEG conc/Sucrose/Time	4	8664	2166	3.49	0.012	*
Error	72	44739	621			
Total	89	512160				

## Appendix 5

Two-Way Analysis of Variance for Percent pollen germination of *Kalanchoe fedtschenkoi* in Robert's *et al.* germinating medium containing 15% sucrose at two temperatures (refer to Fig. 3.3 (a), \*\*\*  $p \leq 0.001$ ; \*\*  $p \leq 0.01$ ; \*  $p \leq 0.05$ ; NS, not significant).

Source	DF	SS	MS	F	P	
Temp.	1	0.05	0.05	0.00	0.954	NS
Time	2	551.29	275.64	17.89	0.000	***
Temp /Time	2	30.62	15.31	0.99	0.385	NS
Error	24	369.73	15.41			
Total	29	951.69				

## Appendix 6

Two-Way Analysis of Variance for Pollen tube length of *Kalanchoe fedtschenkoi* in Robert's *et al.* germinating medium containing 15% sucrose at two temperatures. (refer to Fig. 3.3 (b), \*\*\*  $p \leq 0.001$ ; \*\*  $p \leq 0.01$ ; \*  $p \leq 0.05$ ; NS, not significant).

Source	DF	SS	MS	F	P	
Temp	1	4695.0	4695.0	9.96	0.004	**
Time	2	35186.7	17593.4	37.33	0.000	***
Temp /Time	2	5401.8	2700.9	5.73	0.009	**
Error	24	11309.7	471.2			
Total	29	56593.4				

## Appendix 7

Two-Way Analysis of Variance for The effect of Zn upon percent pollen germination of *Kalanchoe fedtschenkoi* in Robert's *et al.* germinating medium containing either 0, 10, 20, 30, 40 or 50 ppm zinc (as ZnSO<sub>4</sub>). (refer to Fig. 3.4, \*\*\*  $p \leq 0.001$ ; \*\*  $p \leq 0.01$ ; \*  $p \leq 0.05$ ; NS, not significant).

Source	DF	SS	MS	F	P	
Zn Conc	5	3647.97	729.59	18.75	0.000	***
Time	2	2994.04	1497.02	38.47	0.000	***
Zn Conc /Time	10	1622.98	162.30	4.17	0.001	**
Error	36	1400.82	38.91			
Total	53	9665.83				

## Appendix 8

Two-Way Analysis of Variance for The effect of zinc upon pollen tube length of *Kalanchoe fedtschenkoi* in Robert's *et al.* germinating medium containing either 0, 10, 20, 30, 40 or 50 ppm zinc (as ZnSO<sub>4</sub>). (refer to Fig. 3.5, \*\*\*  $p \leq 0.001$ ; \*\*  $p \leq 0.01$ ; \*  $p \leq 0.05$ ; NS, not significant).

Source	DF	SS	MS	F	P	
Zn Conc	5	48112	9622	24.86	0.000	***
Time	2	118855	59428	153.52	0.000	***
Zn Conc /Time	10	10793	1079	2.79	0.0012	*
Error	36	13936	387			
Total	53	191696				

## Appendix 9

Two-Way Analysis of Variance for Percent pollen germination of two genotypes *Brassica rapa* (*Self-compatible* and *dwf2*) in Robert's *et al.* germinating medium containing 15% sucrose at pH 8.0 (refer to Fig. 3.6 (a), \*\*\*  $p \leq 0.001$ ; \*\*  $p \leq 0.01$ ; \*  $p \leq 0.05$ ; NS, not significant).

Source	DF	SS	MS	F	P	
Genetic	1	9.88	9.88	0.52	0.486	NS
Time	2	17.52	8.76	0.46	0.642	NS
Genetic/Time	2	17.58	8.79	0.46	0.642	NS
Error	12	228.96	19.08			
Total	17	273.94				

## Appendix 10

Two-Way Analysis of Variance for Pollen tube length of two genotypes of *Brassica rapa* (*Self-compatible* and *dwf2*) in Robert's *et al.* germinating medium containing 15% sucrose at pH 8.0 (refer to Fig. 3.6b, \*\*\*  $p \leq 0.001$ ; \*\*  $p \leq 0.01$ ; \*  $p \leq 0.05$ ; NS, not significant).

Source	DF	SS	MS	F	P	
Genetic	1	5195.0	5195.0	13.30	0.003	**
Time	2	12496.4	6248.2	15.99	0.000	***
Genetic/Time	2	1461.3	730.6	1.87	0.196	NS
Error	12	4688.2	390.7			
Total	17	23840.8				

## Appendix 11

One-Way Analysis of Variance for Mean percent pollen viability of ten counts per flower of *Brassica rapa*, *dwf2* (refer to Fig. 3.7, \*\*\*  $p \leq 0.001$ ; \*\*  $p \leq 0.01$ ; \*  $p \leq 0.05$ ; NS, not significant).

Source	DF	SS	MS	F	P	
% pollen viability	9	851.9	94.7	3.20	0.002	**
Error	90	2662.4	29.6			
Total	99	3514.2				

## Appendix 12

Multiple-Way Analysis of Variance for Percent pollen germination of *Brassica rapa*, *dwf2* in different sucrose and PEG concentrations. (refer to Fig. 3.8 (a, c, e), \*\*\*  $p \leq 0.001$ ; \*\*  $p \leq 0.01$ ; \*  $p \leq 0.05$ ; NS, not significant).

Source	DF	SS	MS	F	P	
Sucrose	2	289.70	144.85	28.91	0.000	***
PEG conc.	2	96.81	48.41	9.66	0.000	***
Time	2	1110.05	555.02	110.77	0.000	***
Sucrose/PEG conc.	4	143.58	35.89	7.16	0.000	***
Sucrose/Time	4	478.35	119.59	23.87	0.000	***
PEG conc./Time	4	843.34	210.83	42.08	0.000	***
Sucrose/PEG conc/ Time	8	1116.65	139.58	27.86	0.000	***
Error	54	270.56	5.01			
Total	80	4349.05				

## Appendix 13

Multiple-Way Analysis of Variance for pollen tube length of *Brassica rapa*, *dwf2* in different sucrose and PEG concentrations. (refer to Fig. 3.8 (b, d, f), \*\*\*  $p \leq 0.001$ ; \*\*  $p \leq 0.01$ ; \*  $p \leq 0.05$ ; NS, not significant).

Source	DF	SS	MS	F	P	
Sucrose	2	13977.9	6938.9	22.38	0.000	***
PEG conc.	2	8219.9	4109.9	13.26	0.000	***
Time	2	87583.8	43791.9	141.25	0.000	***
Sucrose/PEG conc.	4	18764.8	4691.2	15.13	0.000	***
Sucrose/Time	4	8137.9	2034.5	6.56	0.000	***
PEG conc./Time	4	8037.3	2009.3	6.48	0.000	***
Sucrose/PEG conc/ Time	8	6864.0	858.0	2.77	0.012	*
Error	54	16741.2	310.0			
Total	80	168226.8				

## Appendix 14

Two-Way Analysis of Variance for The effect of Zn on percent germination of *Brassica rapa, dwf2* Robert's *et al.* germinating medium containing either 0, 20, 40 or 60 ppm zinc (as ZnSO<sub>4</sub>). (refer Fig. 3.9 (a, c, e), \*\*\*  $p \leq 0.001$ ; \*\*  $p \leq 0.01$ ; \*  $p \leq 0.05$ ; NS, not significant).

Source	DF	SS	MS	F	P	
Zn Conc.	3	1861.12	620.37	148.19	0.000	***
Time	2	183.28	91.64	21.89	0.000	***
Zn Conc./Time	6	76.30	12.72	3.04	0.024	*
Error	24	100.47	4.19			
Total	35	2221.17				

## Appendix 15

Two-Way Analysis of Variance for The effect of Zn upon pollen tube length of *Brassica rapa, dwf2* in Robert's *et al.* germinating medium containing either 0, 20, 40 or 60 ppm zinc (as ZnSO<sub>4</sub>). (refer Fig. 3.9 (b, d, f), \*\*\*  $p \leq 0.001$ ; \*\*  $p \leq 0.01$ ; \*  $p \leq 0.05$ ; NS, not significant).

Source	DF	SS	MS	F	P	
Zn Conc.	3	38583.4	12861.1	55.49	0.000	***
Time	2	26955.3	13477.7	58.15	0.000	***
Zn Conc./Time	6	8965.0	1494.2	6.45	0.000	***
Error	24	5562.7	231.8			
Total	35	80066.4				

## Appendix 16

Two-Way Analysis of Variance for The effect of copper upon percent pollen germination of *Brassica rapa, dwf2* in Robert's *et al.* germinating medium containing either 0, 2, 4 or 6 ppm copper (as CuSO<sub>4</sub>). (refer Fig. 3.10 (a, c, e), \*\*\*  $p \leq 0.001$ ; \*\*  $p \leq 0.01$ ; \*  $p \leq 0.05$ ; NS, not significant).

Source	DF	SS	MS	F	P	
Cu Conc.	3	557.449	185.816	74.67	0.000	***
Time	2	331.716	165.858	66.65	0.000	***
Zn Conc./Time	6	31.320	5.220	2.10	0.091	NS
Error	24	59.726	2.489			
Total	35	980.211				

## Appendix 17

Two-Way Analysis of Variance for The effect of copper on pollen tube length of *Brassica rapa, dwf2* in Robert's *et al.* germinating medium containing either 0, 2, 4 or 6 ppm copper (as CuSO<sub>4</sub>). (refer Fig. 3.10 (b, d, f), \*\*\* p ≤ 0.001; \*\* p ≤ 0.01; \* p ≤ 0.05; NS, not significant).

Source	DF	SS	MS	F	P	
Cu Conc.	3	5886.4	1962.1	48.23	0.000	***
Time	2	8352.6	4176.3	102.66	0.000	***
Zn Conc./Time	6	2221.2	370.2	9.10	0.000	***
Error	24	976.4	40.7			
Total	35	7436.5				

## Appendix 18

One-Way Analysis of Variance for Pollen tube growth length *in vivo* of *Brassica rapa, dwf2* at 2, 4, 6, 8, 24 and 48 hours after pollination. (refer to Fig. 3.11, \*\*\* p ≤ 0.001; \*\* p ≤ 0.01; \* p ≤ 0.05; NS, not significant).

Source	DF	SS	MS	F	P	
Non-Linear Regression	3	123153.8326	41051.2775	5.5286	0.1570	NS
Residual	2	14850.5549	7425.2774			
Total	5	138004.3875	27600.8775			

Fitted line is a modified hyperbola :  $y = a - \frac{b}{(1 + cx)^d}$  ; R<sup>2</sup> = 0.893

## Appendix 19

One-Way Analysis of Variance for The Effect of Cu on Percent of Pollen Viability in *Brassica rapa, dwf2* (refer to Fig. 3.12, \*\*\* p ≤ 0.001; \*\* p ≤ 0.01; \* p ≤ 0.05; NS, not significant).

Source	DF	SS	MS	F	P	
Treatment	2	656.8	328.4	24.53	0.000	***
Error	18	241.0	13.4			
Total	20	897.7				

## Appendix 20

One-Way Analysis of Variance for Movement of granules within the cytoplasm of pollen tubes of *Hippeastrum cv. Belinda* three hours after incubation commenced at room temperature (ca. 20 °C). (refer to Fig. 3.13 (a), \*\*\*  $p \leq 0.001$ ; \*\*  $p \leq 0.01$ ; \*  $p \leq 0.05$ ; NS, not significant).

Source	DF	SS	MS	F	P
Non-linear Regression	2	1.1198	0.5599	17.9314	0.0018 **
Residual	7	0.2186	0.0312		
Total	9	1.3384	0.1487		

Fitted line is of the equation :  $y = ae \left[ \frac{-0.5(x-x_0)^2}{b} \right] ; R^2 = 0.873$

## Appendix 21

One-Way Analysis of Variance for Movement of granules within the cytoplasm of pollen tubes of *Hippeastrum cv. Appleblossom* three hours after incubation commenced at room temperature (ca. 20 °C). (refer to Fig. 3.13 (b), \*\*\*  $p \leq 0.001$ ; \*\*  $p \leq 0.01$ ; \*  $p \leq 0.05$ ; NS, not significant).

Source	DF	SS	MS	F	P
Non-linear Regression	1	0.5243	0.5243	18.1896	0.0027 **
Residual	8	0.2306	0.0288		
Total	9	0.7549	0.0839		

Fitted line is of the equation :  $y = y_0 + ax ; R^2 = 0.694$

## Appendix 22

One-Way Analysis of Variance for Movement of granules within the cytoplasm of pollen tubes of *Hippeastrum cv. Appleblossom* in control (0 ppm Zn) three hours after start of incubation. (refer to Fig. 3.14, \*\*\*  $p \leq 0.001$ ; \*\*  $p \leq 0.01$ ; \*  $p \leq 0.05$ ; NS, not significant).

Source	DF	SS	MS	F	P
Non-linear Regression	1	0.5243	0.5243	18.1896	0.0027 **
Residual	8	0.2306	0.0288		
Total	9	0.7549	0.0839		

$R^2 = 0.694$

### Appendix 23

One-Way Analysis of Variance for Movement of granules within the cytoplasm of pollen tubes of *Hippeastrum cv. Appleblossom* in 50 ppm Zn three hours after start of incubation.(refer to Fig. 3.14, \*\*\*  $p \leq 0.001$ ; \*\*  $p \leq 0.01$ ; \*  $p \leq 0.05$ ; NS, not significant).

Source	DF	SS	MS	F	P
Non-linear Regression	1	0.0485	0.0485	171.4286	0.0010 ***
Residual	3	0.0008	0.0003		
Total	4	0.0493	0.0123		

$$R^2 = 0.982$$

### Appendix 24

One-Way Analysis of Variance for Movement of granules within the cytoplasm of pollen tubes of *Hippeastrum cv. Appleblossom* in 100 ppm Zn three hours after start of incubation.(refer to Fig. 3.14, \*\*\*  $p \leq 0.001$ ; \*\*  $p \leq 0.01$ ; \*  $p \leq 0.05$ ; NS, not significant).

Source	DF	SS	MS	F	P
Non-linear Regression	1	0.0209	0.0209	5.8892	0.0936 NS
Residual	3	0.0107	0.0036		
Total	4	0.0316	0.0079		

$$R^2 = 0.662$$

### Appendix 25

One-Way Analysis of Variance for The effect of Zn (as  $ZnSO_4$ ) upon pollen tube growth in two genotypes of *Hippeastrum (cv.Appleblossom; cv. Belinda)* after 5 hours incubation.(refer to Fig. 3.15, \*\*\*  $p \leq 0.001$ ; \*\*  $p \leq 0.01$ ; \*  $p \leq 0.05$ ; NS, not significant).

Source	DF	SS	MS	F	P
Treatments	5	6531678	1306336	15.14	0.000 ***
Error	54	4659516	86287		
Total	59	11191193			

## Appendix 26

One-Way Analysis of Variance for The effect of storage (age) on pollen tube growth length in *Hippeastrum cv. Appleblossom* in 20% sucrose three hours after incubation began. (refer to Fig. 3.16, \*\*\*  $p \leq 0.001$ ; \*\*  $p \leq 0.01$ ; \*  $p \leq 0.05$ ; NS, not significant).

Source	DF	SS	MS	F	P
Non-linear Regression	1	39702.5558	39702.5558	55.0319	0.0853 NS
Residual	1	721.4464	721.4464		
Total	2	40424.0022	20212.0011		

$$R^2 = 0.982$$

## Appendix 27

One-Way Analysis of Variance for The effect of storage (age) on pollen tube growth length in *Hippeastrum cv. Appleblossom* in 50 ppm Zn three hours after incubation began. (refer to Fig. 3.16, \*\*\*  $p \leq 0.001$ ; \*\*  $p \leq 0.01$ ; \*  $p \leq 0.05$ ; NS, not significant).

Source	DF	SS	MS	F	P
Non-linear Regression	1	33992.6290	33992.6290	223.1541	0.0426 *
Error	1	152.3280	152.3280		
Total	2	34144.9571	17072.4785		

$$R^2 = 0.995$$

## Appendix 28

One-Way Analysis of Variance for The effect of storage (age) on pollen tube growth length in *Hippeastrum cv. Appleblossom* in 100 ppm Zn 5 hours after incubation began. (refer to Fig. 3.16, \*\*\*  $p \leq 0.001$ ; \*\*  $p \leq 0.01$ ; \*  $p \leq 0.05$ ; NS, not significant).

Source	DF	SS	MS	F	P
Non-linear Regression	1	9112.2726	9112.2726	246.7027	0.0405 *
Residual	1	36.9363	36.9363		
Total	2	9149.2089	4574.6044		

$$R^2 = 0.996$$

## Appendix 29

One-Way Analysis of Variance for The effect of storage (age) on pollen tube growth length of *cv. Appleblossom* in all treatments ( 20% sucrose, 50 ppm Zn and 100 ppm Zn ) 5 hours after incubation. (refer to Fig. 3.16, \*\*\*  $p \leq 0.001$ ; \*\*  $p \leq 0.01$ ; \*  $p \leq 0.05$ ; NS, not significant).

Source	DF	SS	MS	F	P	
Treatments	8	2499715	312464	7.78	0.000	***
Error	84	3373745	40164			
Total	92	5873460				

