# 國 立 交 通 大 學

# 生物科技學系

# 碩 士 論 文

## 台灣山櫻花種子於休眠至發芽階段

## 種子內亞硫酸基水解酵素活性的變化

**The Dramatic Changes of Sulfatase Activities in *Prunus campanulata* Maxim. Seeds from Dormancy to Germination**

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## 摘　　要

種子休眠是普遍存在許多植物種子的生理現象，種子可以避開不適合發芽的環境或是逆境，增加植株發芽散佈的優勢；但種子休眠的生化、生理機制至今仍不清楚。台灣山櫻花種子具有深度休眠性，種子利用暖、低溫混合層積處理之後可以解除休眠；但山櫻花種子僅經過暖溫層積處理後可能逐漸成熟，但播種在適合的環境之下仍不能發芽。山櫻花種子經過暖溫或冷溫層積處理後，種子內亞硫酸基水解酵素的活性有很顯著的改變。本文說明亞硫酸基水解酵素的活性變化可能對於山櫻花種子的發育及從休眠至解除休眠、發芽過程中扮演重要的調控功能。

# The Dramatic Changes of Sulfatase Activities in *Prunus campanulata* Maxim. Seeds from Dormancy to Germination

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ABSTRACT

Seed dormancy is an ecological adaptation for many plant species to avoid the stress and unfavorable environment of germination. However, there have been very few molecular or biochemical studies about the detail of seed dormancy. *Prunus campanulata* Maxim. (Taiwan Cherry) seeds are deeply dormant. Warm followed by cold stratification, which contains imbibition and warm following cold treatment, can release seeds from dormancy to germination. *P. campanulata* seeds become mature during warm stratification but will not germinate even in optimal condition. Dramatic changes of the sulfatase activities were observed following warm and cold stratification of the *P. campanulata* seeds. Desulfation might be important for the regulation of cherry seeds from dormancy to germination. This report explored desulfation and its effects on dormancy and development of *P. campanulata* seeds.

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I love you all.

Leslie 來

2006.7 新竹

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## ABBREVIATIONS AND SYMBOLS

| | |
|---|---|
| $\varepsilon$ | Absorption (extinction) coefficient |
| $A_{280}$ | Absorption at 280nm |
| $A_{760}$ | Absorption at 760nm |
| AMP | Adenosine -5'-monophosphate |
| C1 | Seeds treated with 1 week cold stratification |
| DTT | Dithiothreitol, Cleland's Reagent |
| EDTA | (Ethylenedinitrilo)tetraacetic acid |
| $K_i$ | Inhibitory constant |
| $K_m$ | Michaelis constant |
| MU | 4-methylumbelliferone |
| MUS | 4-methylumbelliferyl sulfate |
| *P. campanulata* | *Prunus campanulata* Maxim. |
| PAP | Adenosine 3',5'-bisphosphate |
| | 3'-phosphoadenosine 5'-phosphate |
| PAPS | Adenosine 3'-phosphate 5'-phosphosulfate |
| | 3'-phosphoadenosine 5'-phosphosulfate |
| PMSF | Phenylmethylsulfonyl fluoride |
| PST | Phenol sulfotransferase |
| SDS | Sodium Dodecyl Sulfate |
| TEMED | N,N,N',N',-tetramethylethylene diamine |
| Tris | 2-Amino-2-(hydroxymethyl)-1,3-propanediol |
| $V_{max}$ | Maximum velocity |
| W1 | Seeds treated with 1 week warm stratification |

**ABBREVIATIONS AND SYMBOLS (continued)**

| | |
|---|---|
| W1C4 | Seeds treated with 1 week warm then 4 weeks cold stratification |
| W1C6 | Seeds treated with 1 week warm then 6 weeks cold stratification |
| W6C4 | Seeds treated with 6 weeks warm then 4 weeks cold stratification |
| W6C6 | Seeds treated with 6 weeks warm then 6 weeks cold stratification |
| W6C8 | Seeds treated with 6 weeks warm then 8 weeks cold stratification |

# Chapter 1 Introduction

## 1.1 Seed dormancy

Seed dormancy is defined as the failure of an intact viable seed to complete germination under favorable conditions (Bewley, 1997) as an ecological adaptation (Geneve, 2003). The role of seed dormancy is across all species to spread germination across time but in synchrony with seasonal cycle to avoid unfavorable weather to maximize competitive advantage and ensure the establishment of plants (Baskin and Baskin, 1998). Environmental stimuli can trigger dormancy release. Seed moisture content, light, and temperature are arguably the most important on primary dormancy and considerations for conducting and interrupting seed dormancy research (Geneve, 2003). Seed dormancy also depends on seed structures, especially those surrounding the embryo, and on factors, including several plant hormones, affecting the growth potential of the embryo (Koornneef *et al*., 2002) (Figure 1). Studies with model species that have comparatively shallow dormancy have identified a number of genes involved in the regulation of dormancy and germination (Bewley, 1997; Koornneef *et al*., 2002). However, there have been very few molecular studies in more deeply dormant species in which moist seeds require prolonged exposure to low temperature to release dormancy and induce germination (Stephen *et al*., 2003).

There is little is known about the mechanism in detail of seed dormancy and germination (Bewley, 1997).

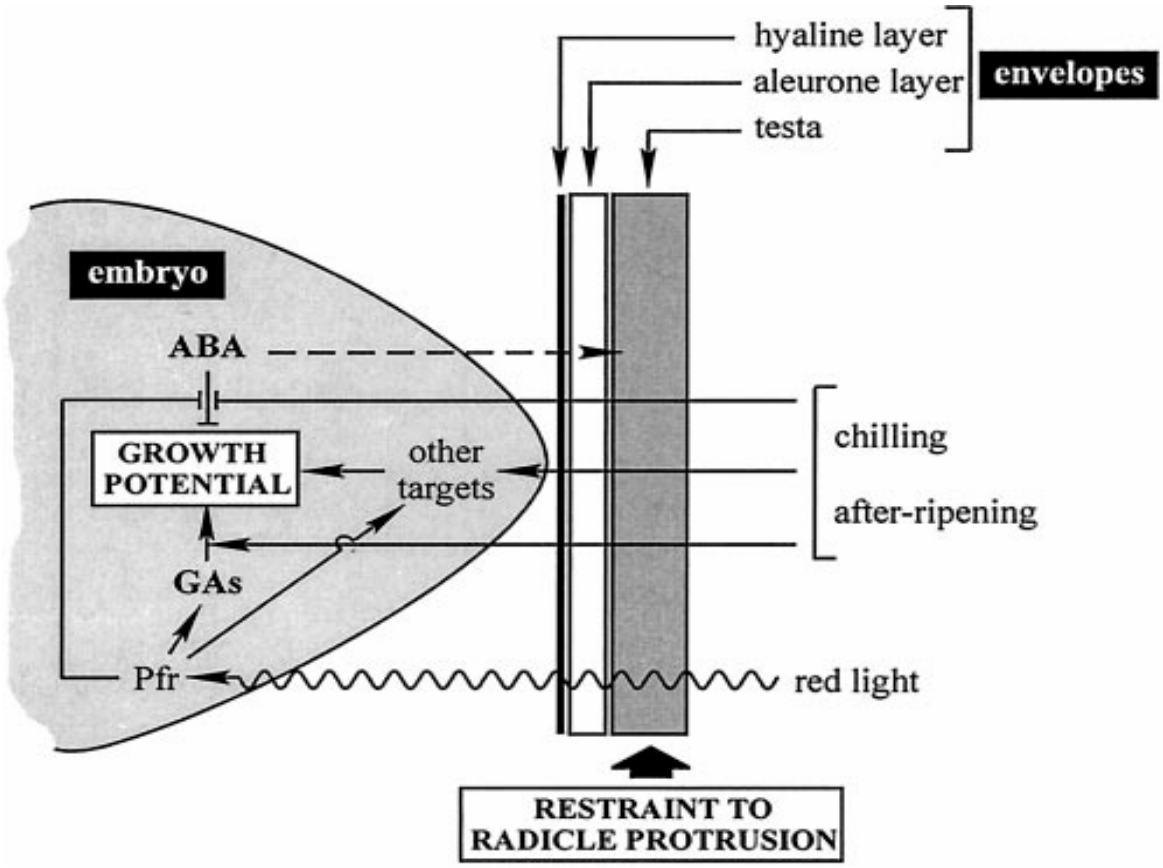


**Figure 1** Seed dormancy is controlled by environmental factors and seed physiology (Debeaujon and Koornneef, 2000).

## 1.2 Seed dormancy of cherry *Prunus campanulata* Maxim.

Deeply dormant Taiwan cherry (*Prunus campanulata* Maxim.) seeds are used to study the seed dormancy. *P. campanulata* seeds are known for their morphological and physiological dormancy which can be broken by a combination of

warm and cold stratification, however some ungerminated seeds were found when seeds were stratified at 5 ℃ alone (Chien *et al.*, 2002).

*P. campanulata* distributed from Japan's Ryukyu Islands and Taiwan to southern China, is a deciduous and outstanding flowering tree with dense branching (Hiroyoshi, 1993). The trees grow on mountains at elevations of between 400 and 2,500 m. *P. campanulata* produces red and campalunate flowers from January to March and dark red fruits from April to May (Figure 2), make it a valuable ornamental plant. The mature fruits of *P. campanulata* exhibit a dark red or dark purple color (Chien *et al.*, 1996; Chien *et al.*, 2002) (Figure 3).

The dormancy mechanism in *Prunus*, like those of other species of deeply dormant seeds, appears to be complex. *Prunus* seeds have embryo dormancy and require a period of after-ripening in the presence of moisture and oxygen to overcome it (Grisez *et al.*, 2000). The embryo is dormant and the surrounding structures (endosperm, seed coat and endocarp) also impose some control (coat-enhanced dormancy). Embryo dormancy is deemed to be present when the embryo fails to grow after the removal of the tissue that encloses it (Stephen *et al.*, 2003). In the other hand, because of their stony endocarps, *Prunus* seeds often been thought to have seedcoat dormancy. The surrounding hard endocarp of embryo appeared to a weakening to permit radicle protrusion after stratification in *P. campanulata* (Chien *et*

*al.*, 2006), but it is permeable to water. *Prunus* is not thought truly hard-seeded (Grisez *et al.*, 2000).

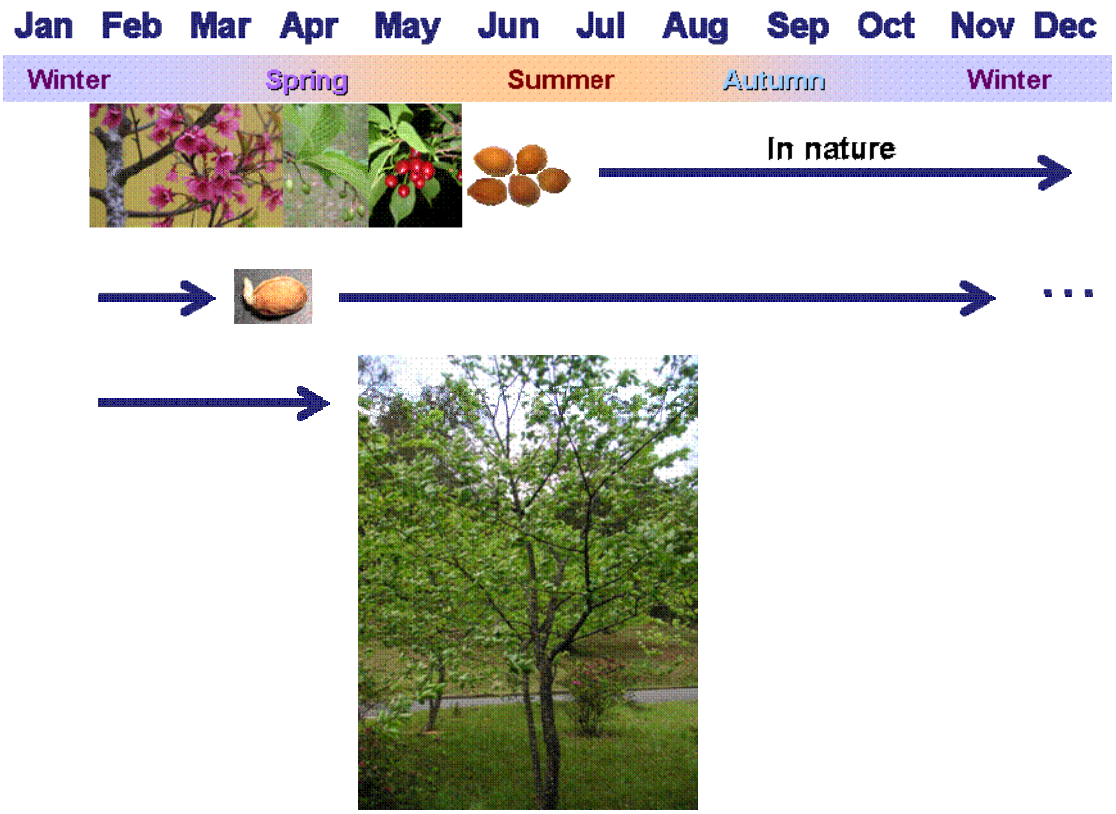


**Figure 2** The life history of *Prunus campanulata* Maxim. in nature.


**Figure 3** Flowers, fruits, and seeds of cherry *Prunus campanulata* Maxim.

Development, maturity and inhibition of germination may be involved in seed dormancy. Lee *et al*. (2006) exposed that significant protein changes between dormant and dormancy-broken seed of *P. campanulata* which were observed by 2D gel electrophoresis. There are some biochemical reactions activated to maintain or break seed dormancy.

## 1.3 Biochemistry about seed dormancy

The direct enzymetic effect or mechanism about seed dormancy remains to be answered. Little is known regarding their roles or interactions with other observed changes although many enzymes have been studied with respect to dormancy. Lang (1994) reviewed seed dormancy investigation of enzymology in seed physiology. Membrane lipid biosynthetic enzymes were thought to be prime targets for low-temperature regulation because biochemical and physical membrane restructuring usually occurs with exposure to low temperature. Seeds are particularly rich in proteases used to hydrolyze storage proteins during the last stages of germination and during embryonic development (Gegenheimer, 1990).

Some biomolecules about seed dormancy, germination and plant development are reported. Debeaujon *et al.* (2000) indicated that most of the pigmentation mutants exhibited a reduced dormancy compared with corresponding wild-type of

*Arabidopsis*. Dormancy of cereal caryopses might be at least partially controlled by the high level of free phenolic acid (Weidner and Paprocka, 1997). Some flavonoid conjugates, including the sulfate esters, may play a role in the regulation of plant growth by blocking the quercetin-stimulated accumulation of the auxin phytohormone (Faulkner and Rubery, 1992). The sulfated peptide phytosulfokine (PSK, Tyr($SO_3H$)-Ile-Tyr($SO_3H$)- Thr-Gln) was an intercellular signal that plays a key role in cellular differentiation and proliferation in plants (Matsubayashi *et al.*, 2002). Desulfated PSK lost both mitogenic activity and competitive ability for ligand binding (Matsubayashi and Sakagamin, 1999). The activity of tyrosylprotein sulfotransferase (TPST) involved in PSK biosynthesis of higher plant was detected and an *in vitro* assay system of TPST was established (Hanai *et al.*, 2000).

## 1.4 Biological functions of sulfation and desulfation in plants

Some biomolecules are activated by sulfation or desulfation in plants, sulfation and desulfation may play an important role in seed dormancy, germination and plant development. It was thought that sulfation and desulfation may play the part of the intermolecular recognition and signaling processes in plant growth, development, and adaptation to stress (Varin *et al.*, 1997). The sulfation (also known as sulfonation and sulfuryation) of biomolecules have long been known to take place

in a variety of organisms, and new biological functions continue to be uncovered in connection with these important transformation (Chapmen *et al*., 2004). Sulfonate conjugation not only facilitates transport and excretion of hydrophobic molecules by increasing their water solubility, it abolishes the biological activity of hormones such as estrogens. The sulfonation reaction also can lead to the production of biologically active molecules (Gidda *et al*., 2003). Sulfotransferases (EC 2.8.2.*), the enzymes that catalyze sulfation, are involved in detoxification, hormone regulation, drug metabolism, molecular recognition, and biochemical signal pathway, and implicated in many pathophysiological processes (Chapmen *et al*., 2004, Figure 4).

Sulfatases (EC 3.1.5.*) cleave sulfate esters in biological systems. Sulfatases are underlined by the presence of several genetic disorders characterized by the abnormal accumulation of intermediate sulfate compounds in human (Parenti *et al*., 1997). Sulfate conjugation constitutes an important reaction in the transformation of xenobiotics and in the modulation of the biological activity of steroid hormones and neurotransmitter in mammals (Varin *et al*., 1997). Because they have been implicated in the outset of hormone-dependent cancers, lysosomal storage disorders, developmental abnormalities, and bacterial pathogenesis, sulfatases were considered to be targets for therapeutic endeavors (Figure 5).

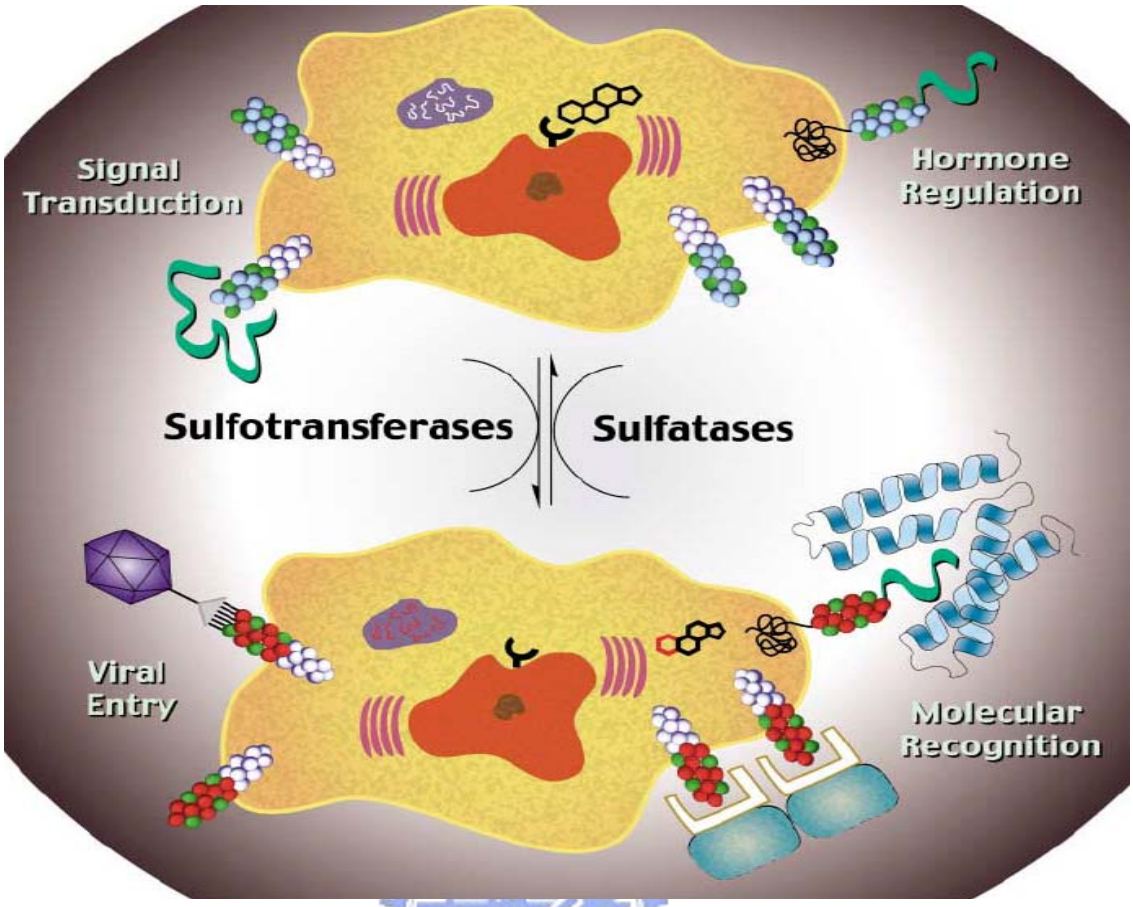


**Figure 4** The biological function of sulfotransferases (Chapmen *et al*., 2004).

Desulfation activity in plants has seldom been reported in the literatures. Arylsufatases in the unicellular green alga *Chlamydomonas reinhardtii* (de Hostos *et al*., 1988; Yildiz *et al.*, 1994) and the multicellular green alga *Volvox carteri* (Hallmann and Sumper, 1994) were produced in response to sulfate deprivation in environment and to develop the capacity to transport sulfate more rapidly. Baum and Dodgson (1957) detected the arylsulfatase activity in the seeds of *Sinapis niger* (black mustard), but no further investigation for the biochemical properties and biological

function of arylsulfatase from *Sinapis niger*. Roots of sterile –grown crops contained arylsulfatase activity (Knauff *et al.*, 2003). It was quantified within the rhizosphere and roots of different crop species. It was suggested that arylsulfatase contributed to the mineralization of organic sulfur to sulfate for plant uptake, and arylsulfatase activity was also induced under sulfate deficiency condition. They also assumed that the enzyme activity is derived from endophytic bacteria not by higher plants, or an alternative possibility is the existence in both plant and bacteria arylsulfatase.

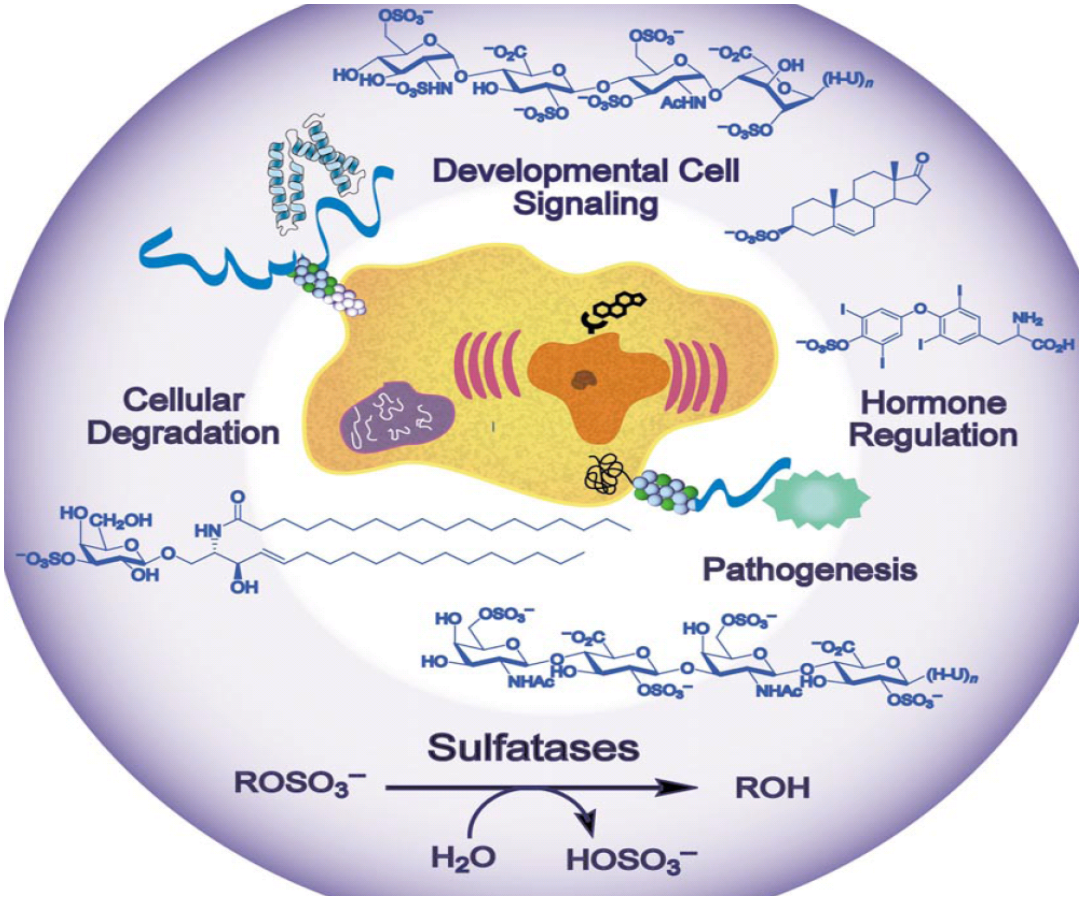


**Figure 5** The biological function of sulfatases (Hanson *et al.*, 2004).

This is the first research about desulfation in higher plant seed dormancy, and some biomolecules were activated by sulfation or desulfation affecting seed dormancy, germination, or seedling development of plants. Using fluorescence photometry assay, we investigated the variation of sulfatase activity in *P. campanulata* seeds following warm and cold stratification. We also present a hypothesis about the regulation of sulfation/desulfation in seeds from development to dormancy in order to explain the mechanism of seed dormancy and related events about sulfation/desulfation in seeds.

# Chapter 2 Materials & Methods

## 2.1 Materials

The RC DC protein assay kit was obtained from BioRad (Richmond, CA, USA). All the other chemicals were the highest purity grade, purchased from Sigma (St. Louis, MO), and the details were listed in Appendix B.

## 2.2 Seed harvesting, the warm and/or cold stratification, and germination test of *P. campanulata* seeds

Mature fruits of *P. campanulata* were harvested in early May every year. Fruits in 2004 were from Ali Mt. (23°32'N, 120°47'E) at an elevation of 2000 m, Chiayi County, central Taiwan. Fruits in 2005 were from Coaling Shihbi park in Yunlin County. Immature fruits of *P. campanulata* were harvested in the interval from March to early May in 2006, from Hsin-hsien nursery, Taipei county. The seeds were extracted by removing the pulp in water, and filled, sunken seeds were used for subsequent treatment. Fresh *P. campanulata* seeds were mixed with moist sphagnum in a polyethylene bag (0.04 mm in thickness) and warm-stratified at alternating temperatures of 30/20 ℃ with 12 hours of fluorescent light (80-100μE/$m^2$/s), and cold-stratified at temperatures of 4℃ in dark. Excess water in

the sphagnum was removed to obtain a water content of about 400% by mass (dry weight basis). For warm plus cold treatment, fresh seeds were stratified at 30/20 ℃ for a period followed by cold stratification.

Germination test was performed at the same temperature as that of the warm stratifying temperature of 20/30 ℃ with 12 hours daily photoperiod. For each test, 3 replicates of 50 seeds each per treatment were used. Germination, judged by radicle protrusion of at least 5 mm, was recorded weekly. Results were expressed as the percentage germination (%).

Fresh and treated seeds were lyophilized and stored at -80℃ for enzyme assay.

## 2.3 Extract and assay of plant sulfatases in *P. campanulata* seeds

The endocarp, testa or embryo of *P. campanulata* seeds was homogenized with PRO 250 homogenizer (PRO Scientific, Oxford, CT, USA) contained 125 mM sucrose, 1 mM dithiotheitol, 1 mM EDTA , 10%(v/v) glycerol, 1 mM PMSF, and 10 mM Tris-HCl at pH 7.4. The suspension was centrifuged for 30 minute at 22,000 g at 4℃ twice. The final supernatant liquid was used in assaying of sulfatase/sulfotransferase activity with fluorescence spectrometry as describe below.

The sulfatase assaying reaction mixture (1000 μl) contained 100 mM

Tris-base (pH 7.0) or 100 mM phosphate buffer (pH 7.0), 5 mM 2-mercaptoetnol, 500 μM MUS, and *P. campanulata* seed extract, incubated in 25 ℃. The difference of fluorescence 450 nm emission excited at 350 nm was detected to calculate the activity of sulfatases from *P. campanulata* seed extract. The sulfotransferase assay reaction condition is the same plus 10 μM PAP and 5 μM 2-naphthol (Figure 6).

Protein concentrations of *P. campanulata* seed extract were estimated by RC DC protein assay kit from BioRad.

The small molecules in seed extract were removed by passing extract through the HiTrap desalting column. Desalted seed extract was concentrated by ultrafiltering with Amicom Ultra-15 centrifugal filter devices PL-30(30000 nominal molecular weight limit). Desalted seed extract was also used in assaying of sulfatase/sulfotransferase activity.

The experimental flow chart was shown in Figure 7.

## Chapter 3 Results

### 3.1 Germination test of *P.* campanulata seeds

Table 1 is germination percentage of *P. campanulata* freshly harvested seeds or seeds treated by warm or/and cold stratification in three years. The period of every germination test was 12 weeks. Untreated freshly harvested seeds germinated less than 1% each year. Seeds treated with warm stratification also germinated poorly. For example, seeds treated with warm stratification for 6 weeks germinated 0.7% (2003), 6.0 % (2004) and 0.7(2005) in three different year. Therefore, warm stratification did not affect germination of *P. campanulata* seeds directly.

Seeds treated with the same cold stratification germinated incoherently. For example, seeds treated with cold stratification longer than 8 weeks germinated 89.3% (8 weeks, 2003), 12.0%(8 weeks, 2004) and 68.0%(9 weeks, 2005) in three different year. However, the longer cold stratification, the more germination of *P. campanulata* seeds in the same year (Figure 8).

The mixed (warm plus cold) stratification was a better strategy of breaking dormancy of *P. campanulata* seeds. Seeds treated with 6 weeks warm and 8 weeks cold stratification germinated completely. Seeds treated with cold stratification 8 weeks resulted only 12% germination, but seeds treated with 1 week warm and 8

weeks cold stratification germinated completely in 2004. Seeds treated with W1C4 resulted 57.3％ germination, and the germination of seeds treated with W1C6 increased to 94.0％. Familiarly, mixed stratified seeds treated with the same warm but longer cold stratification resulted higher germination percentage. For example, seeds treated with W6C4, W6C6 and W6C8 resulted germination to 52.7％, 72.0%, and 98.0％ in 2004.

3.2 The activity of sulfatases during the progress of dormancy breaking in of *P. campanulata* seeds

The freshly-harvested or treated seeds were separated into endocarp, testa (including seed coat and endosperm), and embryo (including cotyledons, epicotyl, hypocotyl and radicle) (Figure 9), and sulfatase activity was assayed. Sulfatase activity was detected in endocarp and testa of treated seeds, but not in embryo (Figure 10).

Sulfatase activity was not detected in freshly-harvested *P. campanulata* seeds. Different sulfatase activities were recorded in *P. campanulata* seeds that were stratified differently and harvested in different years.

After the process of warm and/or cold stratification In *P. campanulata* seeds harvested in 2004 and assays of sulfatases, tendency of change of sulfatases activity in *P. campanulata* seeds was obtained. The sulfatase activity was detected in testa and

endocarp shown in Figure 11 and Figure 12. There is less sulfatase activity assayed in freshly harvested *P. campanulata* seeds. The freshly-harvested dry *P. campanulata* seeds were imbibed and treated by changing temperature in 1 to 3 weeks, and then the activity of sulfatases began to be induced. The higher sulfatase activity exhibited in only warm-stratified seeds. Seeds treated with warm stratification for exact 3 weeks showed a maximum sulfatases activity in testa and endocarp. The sulfatase acitvity was reduced gradually and stable in testa and endocarp following the period of stratification. Figure 13 showed the sulfatase activities in seeds treated with warm stratification only in different years, 2002, 2003, and 2004. The maximum sulfatase activities were exposed in the first to third weeks during warm stratification. Figure 14 showed the sulfatase activities in seeds treated with cold stratification only in different years, 2004 and 2005. The sulfatase activities were induced following the period during cold stratification, but the sulfatase activities of cold-treated seeds were less than activities of warm-treated seeds. According to Figure 11, 12, 13 and 14, the sulfatase activity in testa were higher than that activity in endocarp per milligram of tissue at the same condition of stratification, but the higher total activity was assayed in endocarp of whole tissue generally. When seed dormancy was released by 6 weeks warm stratification following 8 weeks cold stratification, low sulfatase activity was detected in testa and endocarp. Figure 15 showed the activity

of sulfatases of *P. campanulata* seeds exposed to continue warm then cold stratification, and the cold stratification started from the seventh week to the fourteenth weeks. The dash line indicates the germination percentage for the same treatment and time. Seeds treated with 6 weeks warm plus 8 weeks cold stratification germinated to 98%, and sulfatase activity was low but had stable tendency. However, seeds treated with warm stratification only germinated less than 10%, but exhibited maximum sulfatases activity.

# Chapter 4 Discussion

## 4.1 Germination of *P. campanulata* seeds

According to the germination experiments of *P. campanulata* seeds, most of seeds do not germinate under either warm stratification or cold stratification. Warm plus cold stratification could induce seed germination significantly. The result indicated that a combination of warm and cold stratification is necessary for releasing dormancy of *P. campanulata* seeds completely. Small amount of seeds germinated after cold stratification only, and sometimes the germination percentage was over ten percent depending upon the year and the location of the seed harvested (Chien *et al.*, 2000), such as seed harvested from Coaling Shihbi park in 2005. Only cold-stratified seeds germinated differently possibly due to different maturity of freshly-harvested seeds. In practice, a mixture of fruits at different degree of ripeness could be harvested from different trees although the pericarp color of fruits appeared mature (Chien *et al*, 2006). Warm followed by cold stratification were capable of releasing seed dormancy completely and promoting germination. Sometimes cold stratification also stimulated seed germination, but retained about 10-30% ungerminated seeds in the stratification periods. The timing for stratification is different yearly especially at warm period. It is likely that some seeds need more experienced “warm treatment”

before they were delivered from the field to the laboratory or it may just reflect the heterogeneity of the seeds.

## 4.2 The activity of sulfatases/ST during the progress of dormancy breaking in *P. campanulata* seeds

We found that significant change in the activity of desulfation following warm and/or cold stratification during the progress of dormancy breaking in *P. campanulata* seeds. When *P. campanulata* seeds were harvested freshly, there is less sulfatase activity exhibited in seed envelope. The activity of sulfatases was induced when seeds detected the signal of warmness plus moisture. The sulfatase activity exhibited maximum in only warm-stratified period. It is speculated that specific hydrolysis reactions may participate to initial stage of seed dormancy and dormancy breaking. A stable sulfatase activity results in the release of seed dormancy and promotion of germination. The activity of sulfotransferase was less detected within the period of stratification. However, the seed extract from testa and endocarp of freshly-harvested *P. campanulata* seeds inhibited phenol sulfotransferase (rat ST1A1) activity strongly. Stratified seed extract reduced the inhibition on rat ST1A1 (Tsai YY, 2005, unpublished data) (Figure 16). Significant variations in seed proteins were also observed by 2D gel electrophoresis before and after breaking of seed

dormancy in *P. campanulata* (Lee *et al.*, 2006). The warm and/or cold stratification may act as a signal to change germination potential of *P. campanulata* seeds (Chien *et al.*, 2006). Sulfatase activity arose in *P. campanulata* seeds from warm stratification and the desulfation may participate to regulate the mechanism in the initial stage of *P. campanulata* seed dormancy.

## 4.3 Influence of endocarp and tests on *P. campanulata* seed dormancy

In *P. campanulata* seeds, the sulfatases activity was detected in seed envelope including endocarp and testa. Removing both endocarp and seeds coat promoted radicle protrusion promptly, thus a covering-imposed dormancy appeared in *P. campanulata* seeds (Chien *et al.*, 2006). The sulfatases in testa and endocarp may be induced or remain for of physiological reactions for seed dormancy and germination. The testa in higher plant seeds is known a multifunctional tissue which may interfere with dormancy, and also protects the embryo against adverse environmental conditions (Debeaujon *et al.*, 2000). Seed testa may contain some germination inhibitors, such as phenolic compounds and ABA, to cause chemical dormancy (Lin, 1996). An inhibitory nature was considered to locate mainly in the covering tissues of the freshly harvested *P. campanulata* seed (Chien *et al.*, 2006). It was found that high ABA contents were detected in the testa but not in the embryo and cotyledon, and reduced

6~12 times during warm and/or cold stratification; a higher concentration of $GA_4$ was detected in embryo and cotyledon of dormancy-breaking *P. campanulata* seed (Lee *et al.*, 2006). Lee *et al.* also found that protein patterns of testa significantly differed from those of the cotyledon and embryo in dormant and dormancy-breaking *P. campanulata* seeds. Germination experiments also reveal that the removal of testa of *P. campanulata* seed eliminated its dormancy effect (Chien *et al.*, 2006). However, Geneve (2003) indicated that embryos of deep dormant seeds isolated from these seeds either will not germinate or will grow into abnormal seedlings with a dwarf phenotype, termed physiological dwarf. It seems that not only germination inhibitors but also development promoters are contained in seed testa. Furthermore, the higher total sulfatase activity in *P. campanulata* seeds was assayed in endocarp than activity in testa. There may be some biomolecules hydrolyzed from woody endocarp and testa influx into embryo to regulate the seed dormancy. Nevertheless, there was few publication about influence of endocarp on seed dormancy, but endocarp was thought to prevent imbibition and form seed mechanical dormancy. Germination experiments reveal that the removal of endocarp of *P. campanulata* seed eliminated about 20% dormancy effect (Figure 17) (Chien *et al.*, 2006). There was no report about the influence of endocarp on seed chemical dormancy. We detected sulfatase activity in endocarp and testa of treated *P. campanulata* seeds, but not in freshly-harvested seeds

and treated embryo. Desulfation could affect some metabolites in testa and endocarp of *P. campanulata* seeds between dormancy maintenance and releasing.

## 4.4 Dormancy releasing of *P. campanulata* seeds

*P. campanulata* seeds are deeply dormant. They exhibit morphological as well as physiological dormancy due to the undeveloped embryo at the time of fruit collection (Chien *et al.*, 2002). Freshly-harvested and intact seeds of *P. campanulata* required a 1-6 weeks warm followed by 8 weeks cold stratification for complete germination. However, *P. campanulata* seeds treated by just 8 weeks cold stratification explored different germination percentages in different harvesting years and places. Many scientists discussed that how temperatures affect the physiology to release seed dormancy. According to Geneve (2003), in nature, morphophysiological seeds are usually shed from the plant with an undeveloped embryo that requires a warm period to initiate growth inside of seed covering (morphological dormancy) (Table 2). Once the embryo reaches a certain size, it can respond to chilling temperature, which releases physiological dormancy, so seeds usually require warm (>15℃) followed by cold (1 to 10℃) conditions, i.e. embryo development during the warm temperature cycle and breaking physiological dormancy during the chilling cycle. This is not for *P. campanulata* seeds because removal of teata and endocarp

readily release dormancy (Chien *et al.*, 2006) (Figure 17). We could speculate that seeds were treated by warm stratification for embryo development and maturity, and treated by cold stratification for releasing physiological dormancy, but mechanisms are not understood. Lin (1996) considered that physiologically dormant seeds have the low metabolic ability in embryo and need to stratification to remove the germination inhibitor and produce germination promoter. The warm stratification may allow *P. campanulata* to continuous seed development to obtain more proportion of mature seeds in the seed lots (Chien *et al*., 2006). Many proteins in *P. campanulata* seed sections increasing or decreasing following warm stratification, and these proteins may be involved in the post-harvested maturation of seeds (Lee *et al.*, 2006). In the other hand, temperature also impacts dormancy release for seeds with exogenous physical dormancy on seed covering. For instance, some seeds require high temperature or daily fluctuations (>15℃ change) in temperature to allow imbibitions (Baskin and Baskin, 1998; Geneve, 2003). Effects of warm temperature on seeds are not only permeability of seed envelope but also embryo full development.

It is necessary for seed stratification to imbibe moisture. Factor affecting seed dormancy is the interaction between temperature and seed moisture content. Chilling stratification is not effective unless seeds are hydrated (Geneve, 2003). Some catabolic reactions catalyzed by enzymes occur during imbibition. According

to Vertucci (1993), very few enzyme-mediated reactions occur when dry seeds first hydration level (type 1 water). Some catabolic reactions catalyzed by enzymes occur in second hydration level (type 2 water), but seed germination can only be completed if type 5 water is present, although some metabolism necessary for germination (i.e. protein synthesis) occurs in the fourth hydration level (type 4 water) (Figure 18). Lee *et al.* (2006) showed that changes in some group of proteins probably indicate biochemical activities of the *P. campanulata* seed after water uptake (Lee *et al.*, 2006). Water uptake may improve enzyme activity of catabolism such as hydrolases and sulfatases.

The brief hypothesis of the correlation between seed dormancy and desulfation regulation is shown in Figure 19. Seed testa and endocarp contain several metabolites to regulate dormancy and germination. Seeds germination in various treatments implied that germination inhibitors or dormancy inducers were probably increased or induced during warm stratification, and those were decreased or removed during cold stratification (Lee *et al.*, 2006). Sulfatases may be the key to increase the concentrations of dormancy inducers and germination inhibitors. When warm stratification processed, seed germination was inhibited and embryo was getting fully mature. Mature seeds were treated by cold stratification and germination inhibitors in seeds were degraded, and then seeds germinate completely.

## Chapter 5 Conclusion

This is the first report of sulfatase activity in woody plant. Significant change of the sulfatase activity was observed following warm and cold stratification, the treatments for the dormancy breaking of Taiwan cherry (*P. campanulata*) seed. The sulfatase activity exhibited maximum in only warm-stratified period, and Seed germination followed sulfatase activity occurrence. Sulfatase activity was detected, but PST activity was not. However, the activity of rat phenol sulfotransferase was inhibited strongly by the seed extract. The effect of warm and cold stratification on the activities of sulfation/desulfation was first reported and analyzed. It speculated specific hydrolysis reactions which relate to embryo development and maturity may participate to initial stage of seed dormancy and germination. This research provides a novel view to investigate the biological mechanism of seed dormancy.

## Acknowledgement

We appreciate Dr. Ching Te Chien, Ms. Shun Ying Chen and coworkers of Dr. Chien's team to provide seed materials and assistance and guidance about stratification and germination tests.

**Table 1** Germination percentage of *P. campanulata* seeds following warm or/and cold stratification.

1-1 germination before 2003

| ***Treatment (weeks)*** | *Freshly-harvested seeds* | *Warm 6 + Cold 8* | *Warm 6* | *Cold 2* | *Cold 4* | *Cold 6* | *Cold 8* |
|---|---|---|---|---|---|---|---|
| ***Germination (%)** | 0 | 99.3 | 0 | 0.7 | 22.7 | 70.0 | 89.3 |

1-2 germination in 2004

| ***Treatment (weeks)*** | *Freshly-harvested (Warm 0)* | *Warm 1* | *Warm 2* | *Warm 3* | *Warm 4* | *Warm 5* | *Warm 6* |
|---|---|---|---|---|---|---|---|
| ***Germination (%)** | 4.0 | 3.0 | 2.7 | 4.7 | 5.3 | 2.7 | 6.0 |
| *Cold 4* | - | 57.3 | 68.0 | 70.0 | 33.3 | 52.7 | 52.7 |
| *Cold 5* | 5.3 | - | - | - | - | - | - |
| *Cold 6* | 8.7 | 94.0 | 87.3 | 95.3 | 87.3 | 90.0 | 72.0 |
| *Cold 8* | 12.0 | 100 | 100 | 98.7 | 98.7 | 100 | 98.0 |

**Table 1 continued**

1-3 germination in 2005

| ***Treatment (weeks)*** | *Freshly-harvested* | *Warm 6 + Cold 8* | *Warm 6* | *Cold 2* | *Cold 4* | *Cold 6* | *Cold 8* |
|---|---|---|---|---|---|---|---|
| ***Germination (%)** | 0 | 100 | 0.7 | 0.7 | 22.0 | 51.3 | - |

| ***Treatment (weeks)*** | *Cold 1* | *Cold 3* | *Cold 5* | *Cold 7* | *Cold 9* |
|---|---|---|---|---|---|
| **Germination (%)** | 1.3 | 8.7 | 30.7 | 48.0 | 68.0 |

* Germination, judged by radicle protrusion of at least 5 mm, was recorded weekly for a total 8 weeks.

The abbreviation: Warm, seeds were treated by warm stratification; Cold, seeds were treated by cold stratification;

-, no data.

**Table 2** Dormancy categories. *(*Geneve, 2003)

| Dormancy type | Causes of dormancy | Conditions to break dormancy | Representative genera |
|---|---|---|---|
| 1. primary dormancy | | | |
| a. exogenous dormancy | Imposed by factors outside the embryo | | |
| Physical | Impermeable seedcoat | Scarification | *Baptisia, Convolvulus, Gleditsia, Lupinus, Beta, Iris* |
| Chemical | Inhibitors in seed coverings | Removal of seed covering (fruits)<br>Leaching seeds | |
| b. endogenous dormancy | Imposed by factors in the embryo | | |
| Morphological | The embryo is not fully developed, at the time the seed shed from the plant | Warm or cold stratification | |
| Rudimentary | Small undifferentiated embryo | Cold stratification and potassium nitrate | *Anemone, Ranunculus* |
| Linear | Small differentiated embryo < 1/2 size of the seed | Warm stratification and gibberellic acid | *Daucus, Cyclamen, Viburnum* |
| Physiological | Factors within embryo inhibits germination | | |
| Nondeep | Positively photodormant (requires light) | Red light | *Lactuca, primula* |
| | Negatively photoodormant (inhibited by light) | Darkness | *Cyclamen, Nigella* |
| | After-ripen | Short period of dry storage | *Cucumis, Impatiens* |
| Intermediate | Embryos germinates if separate from the seed coat, often responds to gibberellic acid | Moderate periods (up to 8 weeks) of cold stratification | *Aconitum, Cornus, Pinus* |
| Deep | Embryos do not germinate when remove from seedcoat or will form a physiological dwarf | Long periods (> 8 weeks) of cold stratification | *Dictamnus, Euonymus, Prunus, Rhodotypos* |
| c. combinational | Combination of different dormancy, conditions that must be satisfied sequentially | | |
| Morphophysiological | Combination of underdeveloped or rudimentary embryo and physiological dormancy | Cycle of warm and cold stratification | *Asimina, Helleborus, Ilex, Magnolia, Mertensia* |
| Epicotyl | Radicle begins growth when temperature and water permit, but epicotyl is dormant | Warm followed by cold stratification | *Asarum, Paeonia* |
| Epicotyl and radicle (double dormancy) | Radicle and epicotyl require chilling stratification, but radicle is released during first year and then | Cold stratification followed by warm followed by a second cold stratification | *Convallaria, Trillium* |
| Exo-endodormancy | Combination of exogenous and endogenous dormancy conditions. ex. Physical (hard seedcoat) + intermediate physiological dormancy | Sequential combinations of dormancy release treatments. Ex. Scarification followed by cold stratification | *Cercis, Tilia* |
| 2. secondary dormancy | | | |
| a. thermodormancy | After primary dormancy is relieved, high temperature induced dormancy | Growth regulators or cold stratification | *Apium, Lactuca, Viola* |
| b. conditional dormancy | Change in ability to germinate related to time of the year | Chilling stratification | Many species with endogenous dormancy display conditional dormancy |

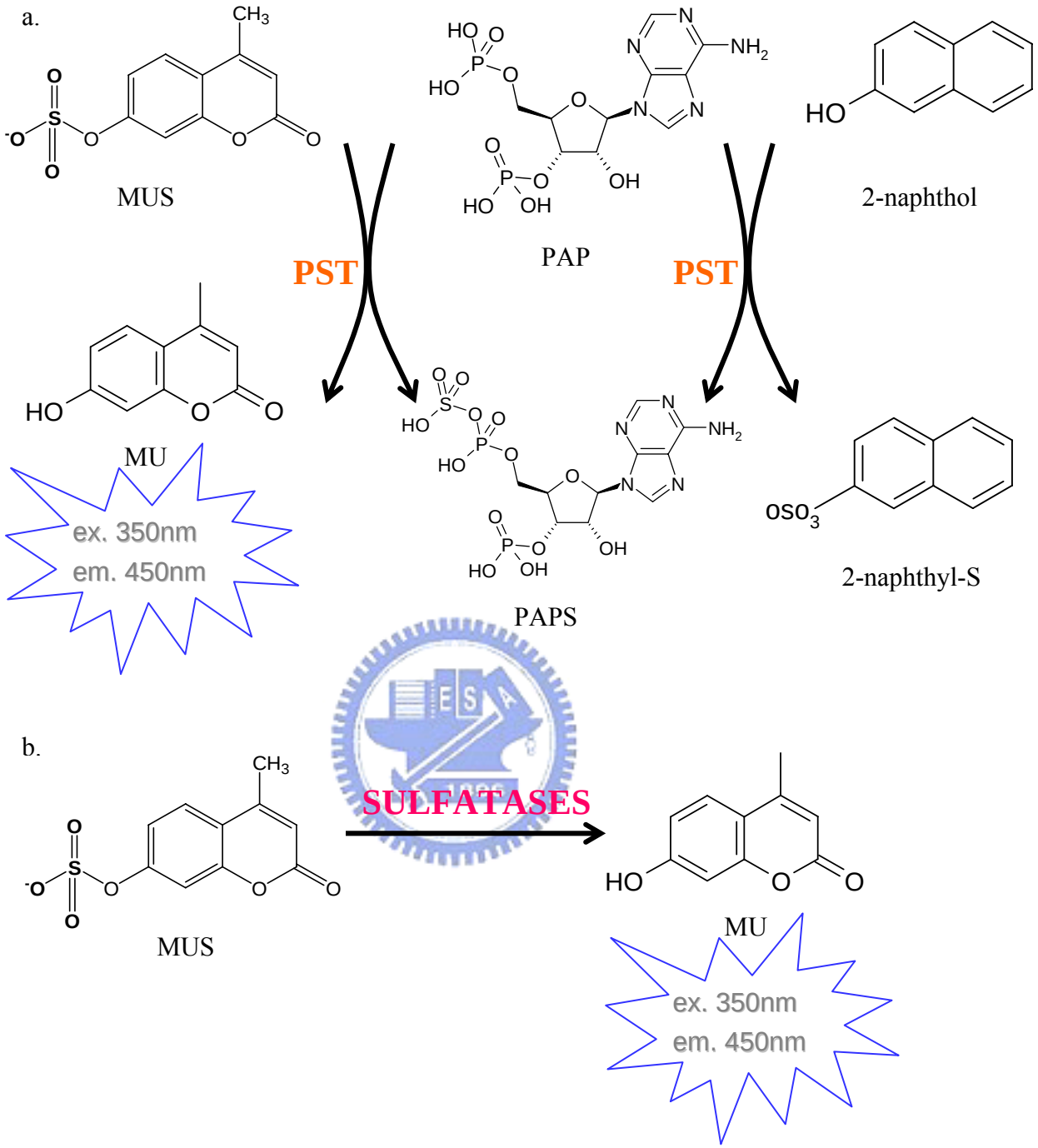


**Figure 6** The reaction of enzyme assay with fluorescence photometry by assaying MU in 350 nm excitation and 450 nm emission. a. The transfer reaction of phenol sulfotransferases (PST). b. The hydolysis reaction of sulfatases.

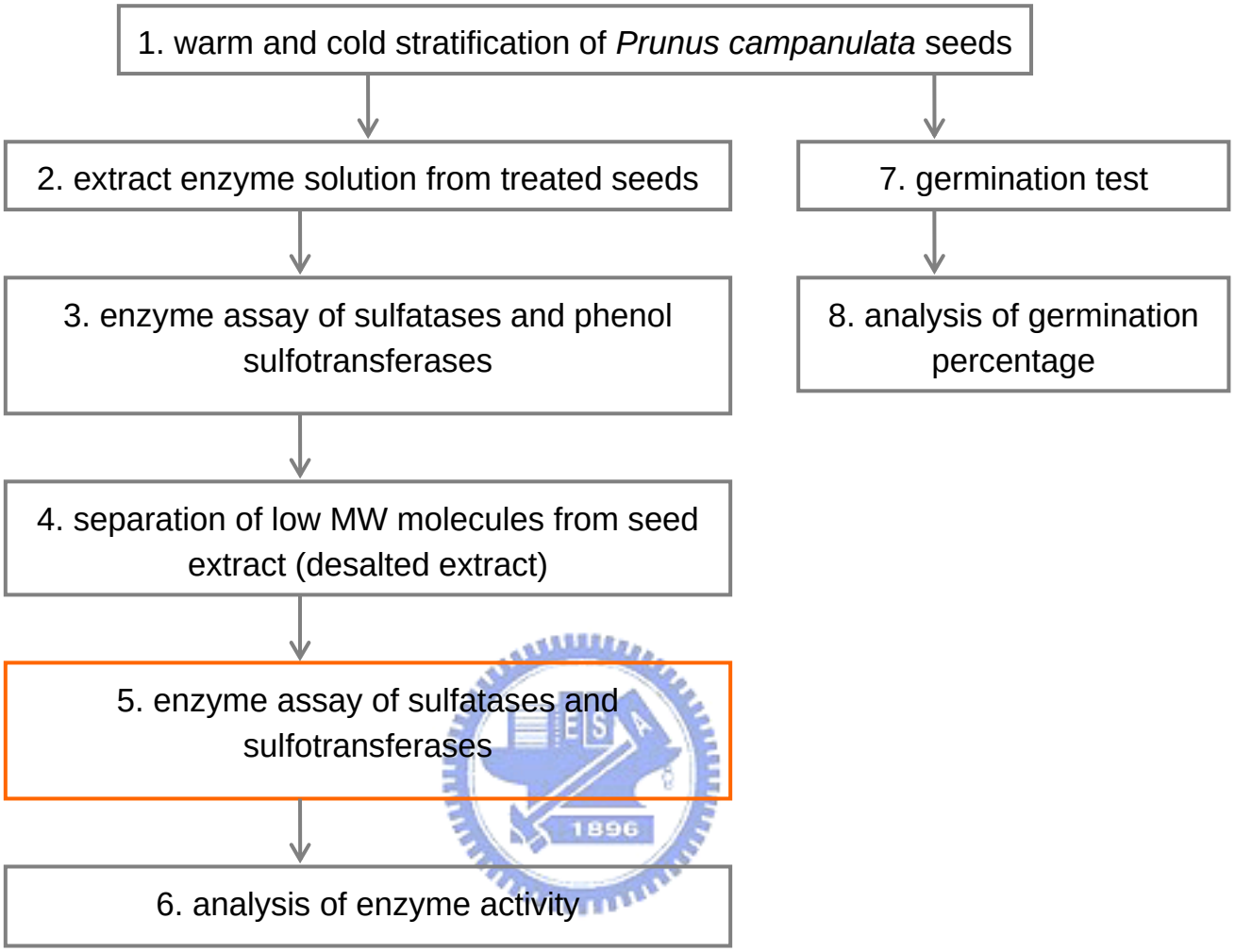


**Figure 7** The overview of experimental flow chart.

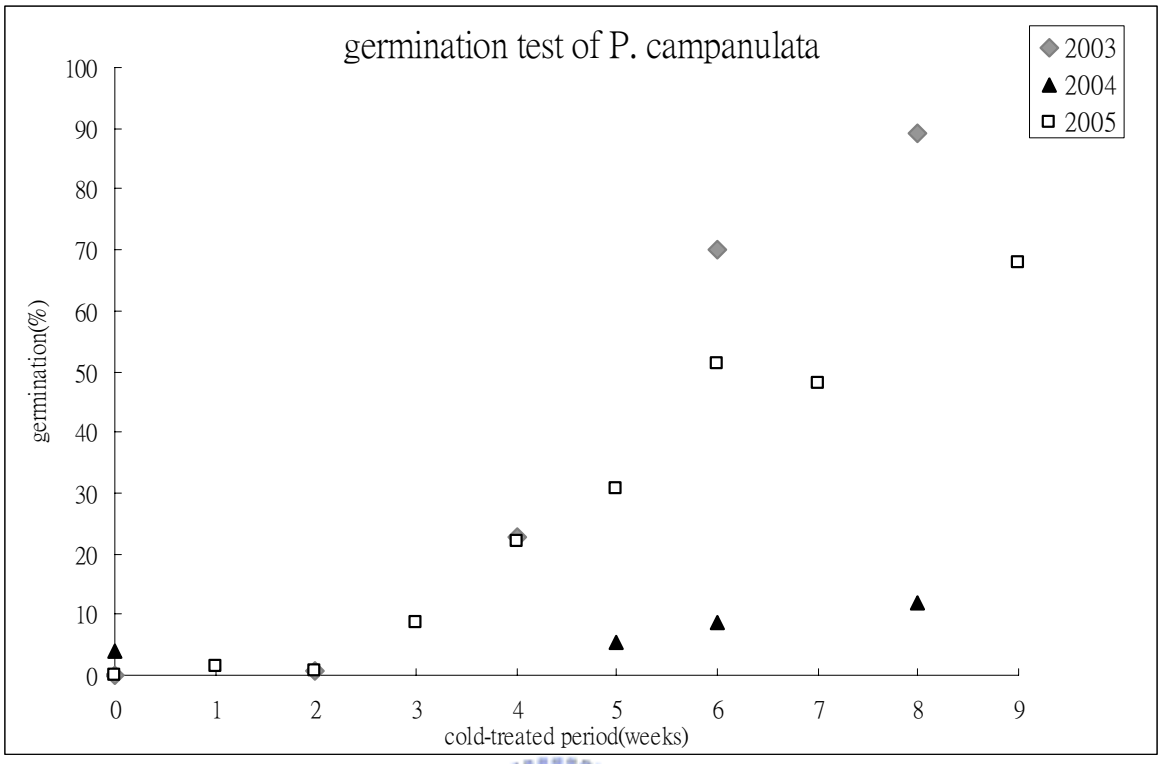


**Figure 8** Germination percentages of *P. campanulata* seed following cold stratification in three different year: before 2003, 2004, 2005.

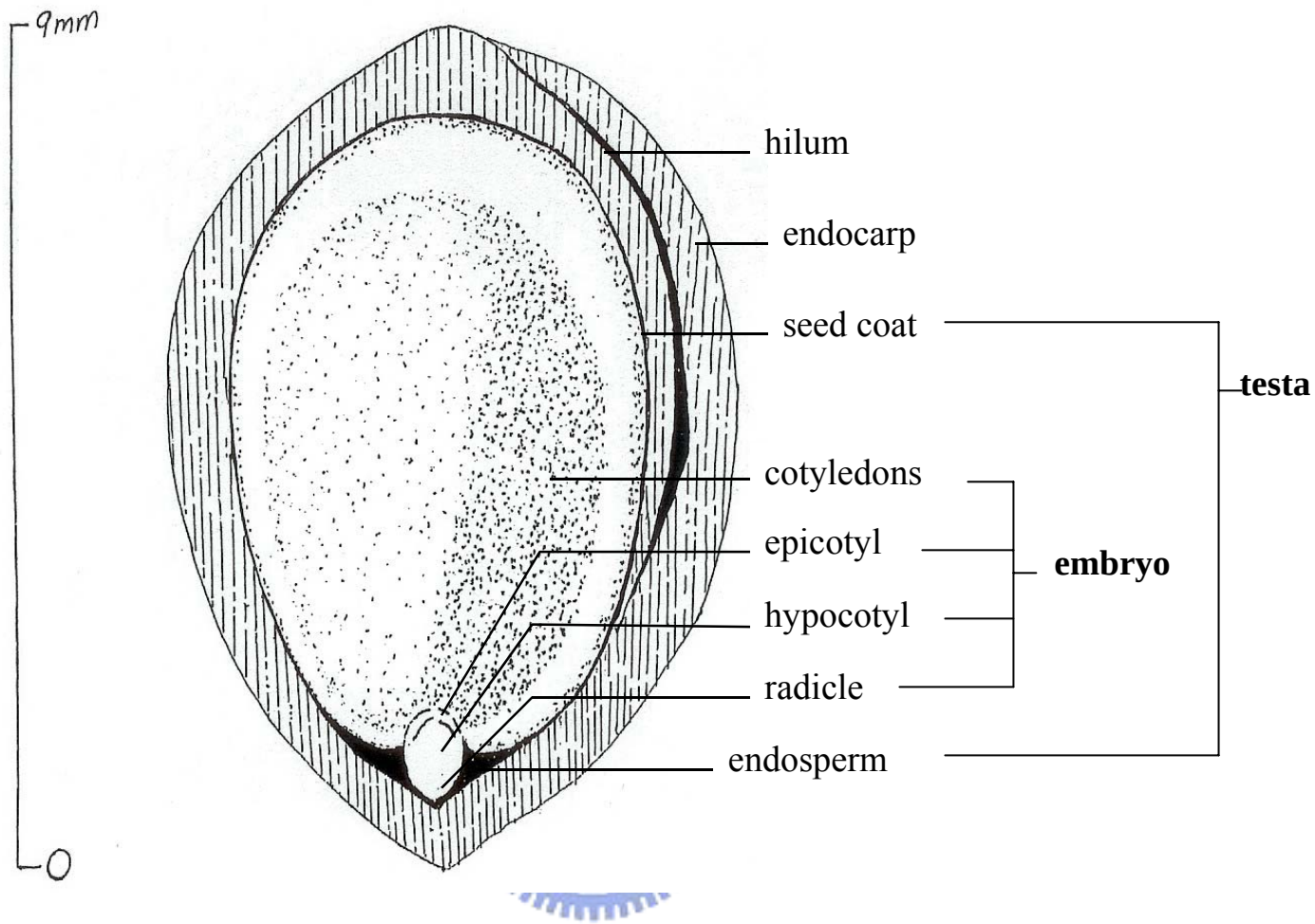


**Figure 9** Longitudinal section through a seed of *P. campanulata*. A small amount of degenerate endosperm can be seen to be adherent to the hypocotyl and radicle (modifying from Chien, *et al.*, 2006).

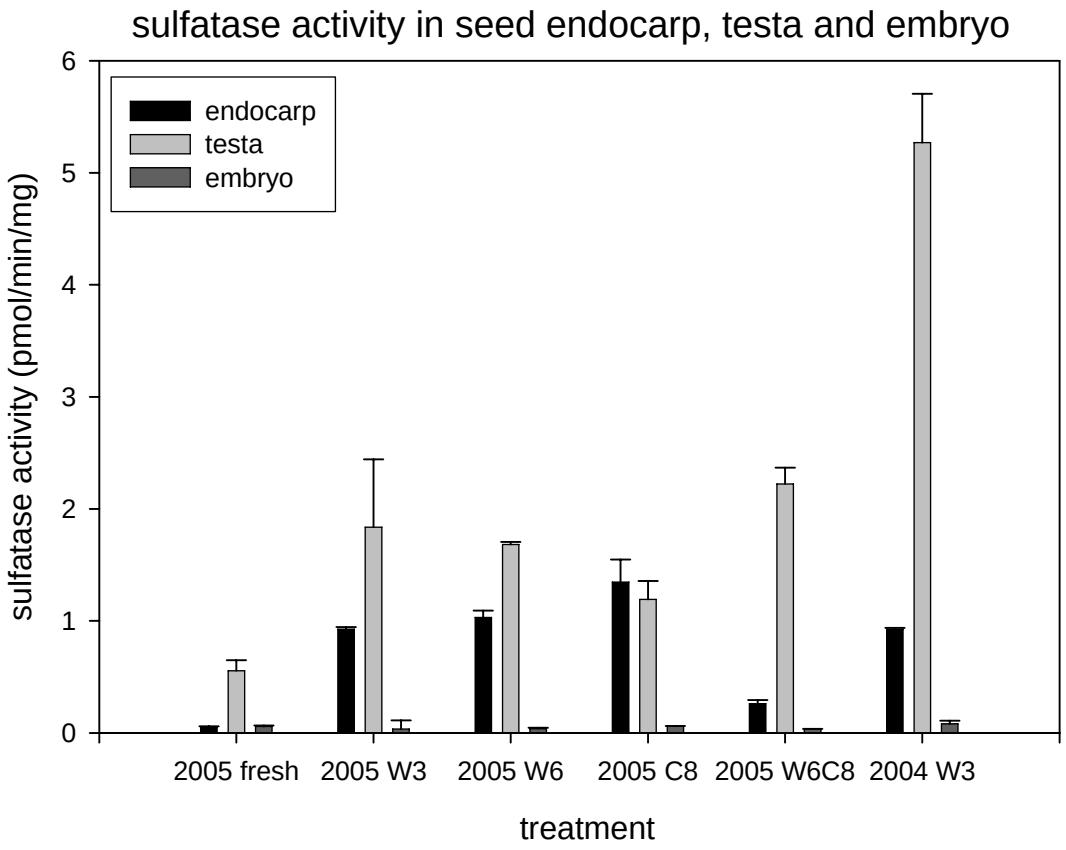


**Figure 10** Sulfatase activity was detected in endocarp, testa , and embryo.

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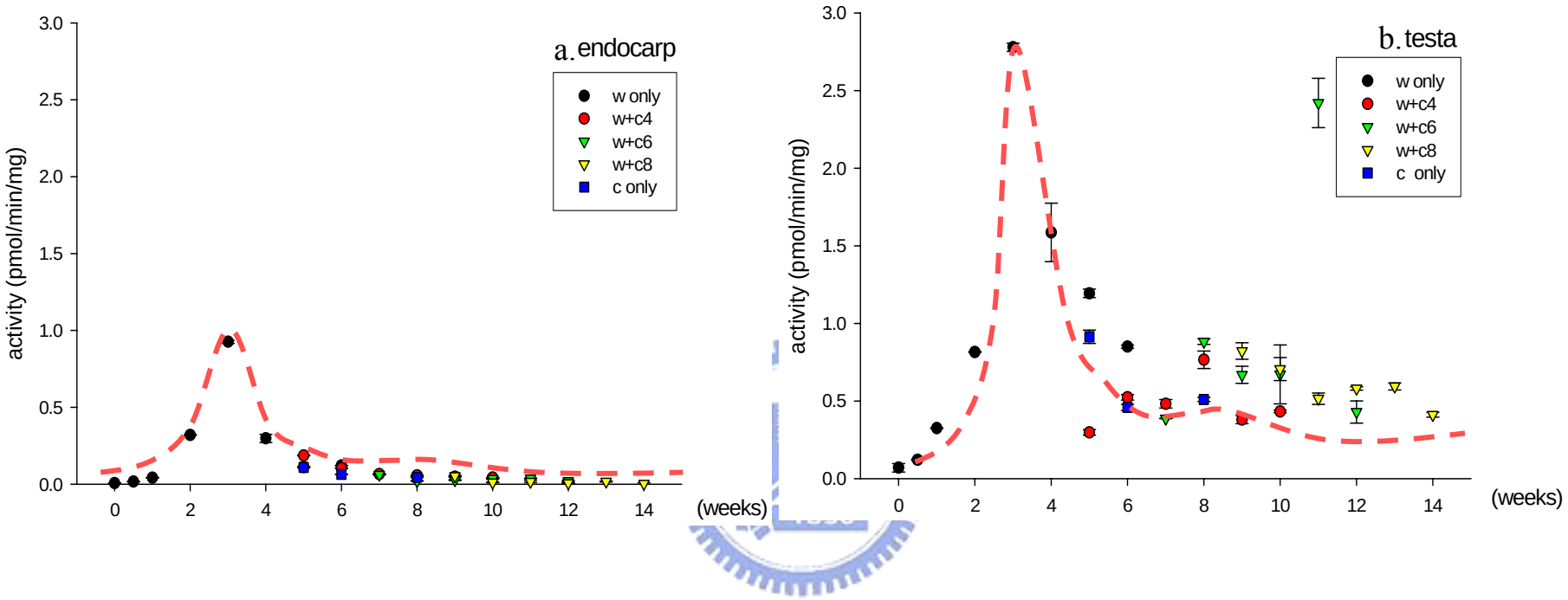


**Figure 11** The activities of sulfatase in *P. campanulata* seed extracts during the warm and/or cold stratification in 2004. a, sulfatase activities in seed endocarp, b, sulfatase activities in seed testa. The x-axis indicates to the sum of the periods of warm and cold stratification (the total stratified time). The y-axis indicates to sulfatase activities detected at different stratifying conditions.

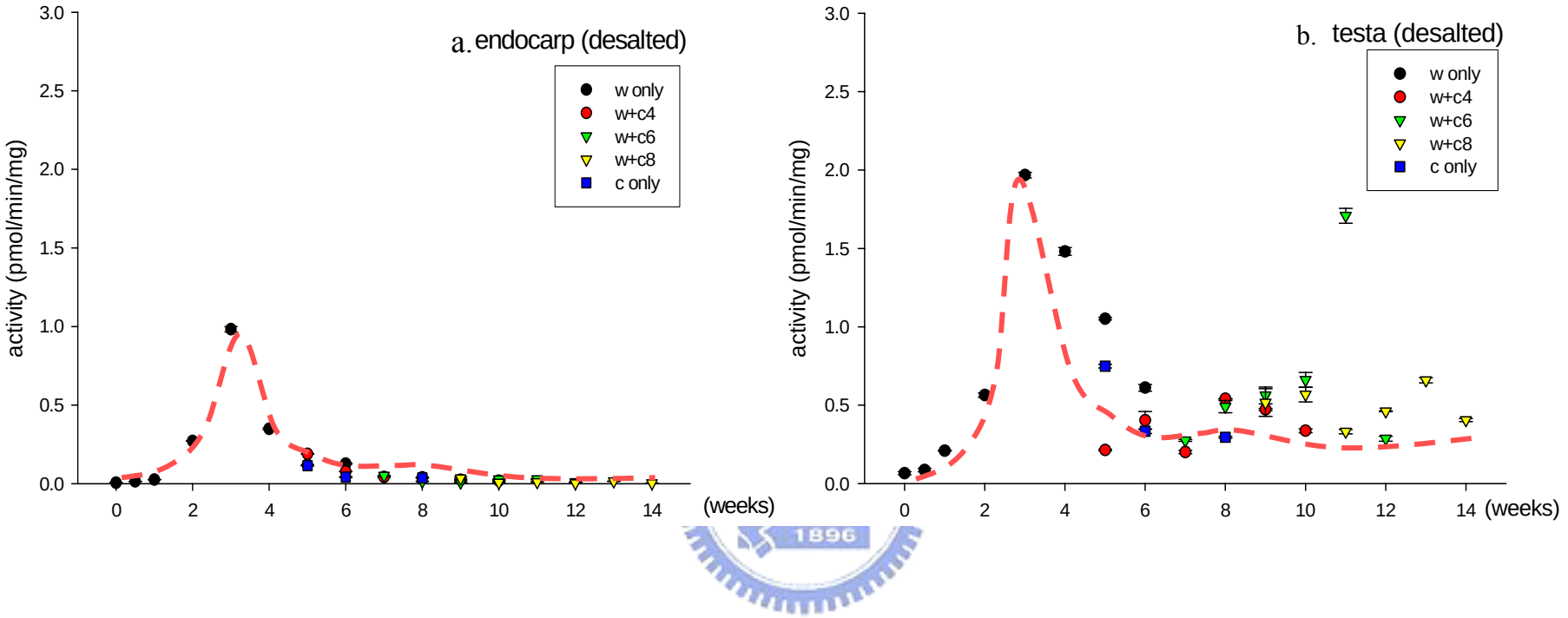


**Figure 12** The activities of sulfatase in *P. campanulata* desalted seed extracts during the warm and/or cold stratification in 2004. a, sulfatase activities in seed endocarp, b, sulfatase activities in seed testa. The x-axis indicates to the sum of the periods of warm and cold stratification (the total stratified time). The y-axis indicates to sulfatase activities detected at different stratifying conditions.

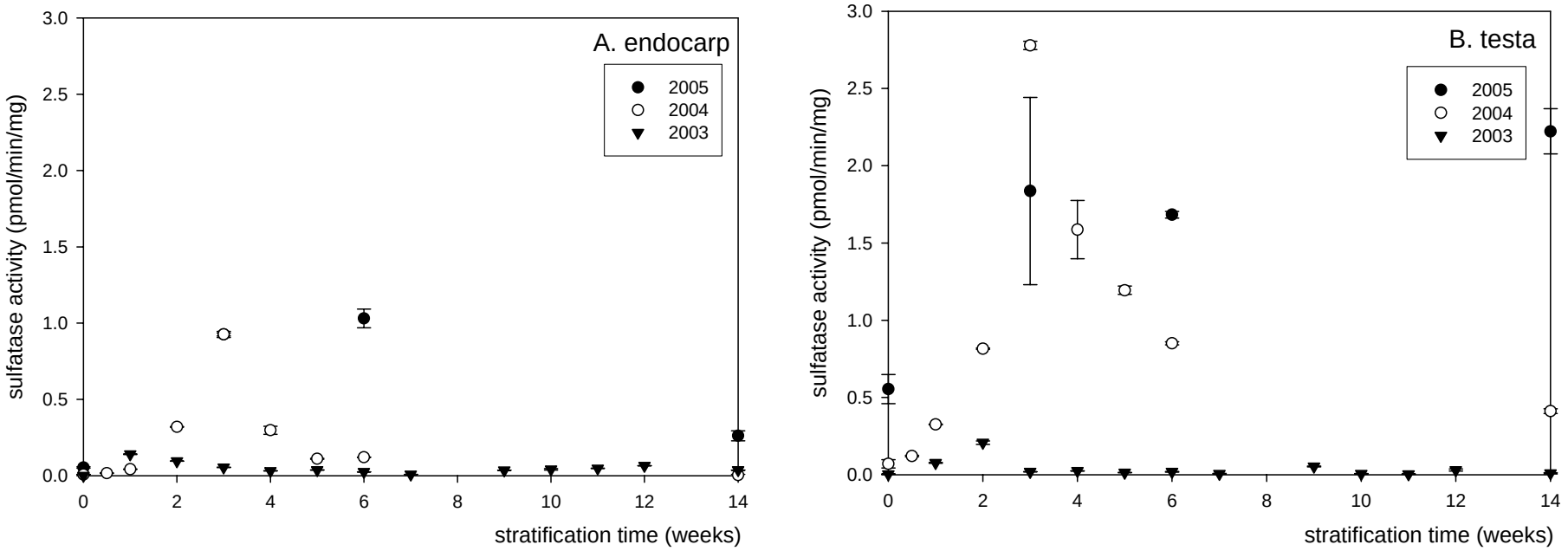


**Figure 13** The tendency of sulfatase activities in *P. campanulata* seed extracts during the warm stratification in 2003(W0 to W12), 2004(W0 to W6) and 2005(W0, W3, W6). The points at the fourteenth week were W6C8 treated seeds. a, sulfatase activities in seed endocarp, b, sulfatase activities in seed testa.

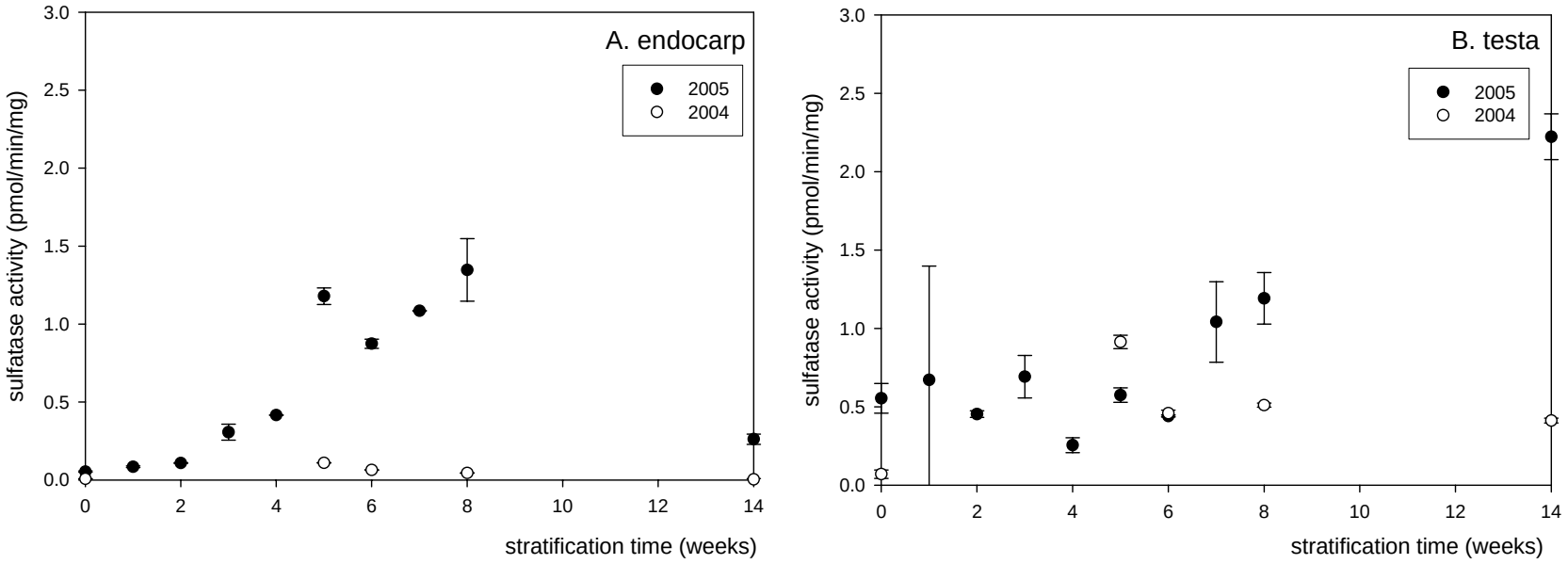


**Figure 14** The tendency of sulfatase activities in *P. campanulata* seed extracts during the cold stratification in 2004(C5, C6 and C8), and 2005 (C0 to C8). The points at the fourteenth week were W6C8 treated seeds. a, sulfatase activities in seed endocarp, b, sulfatase activities in seed testa.

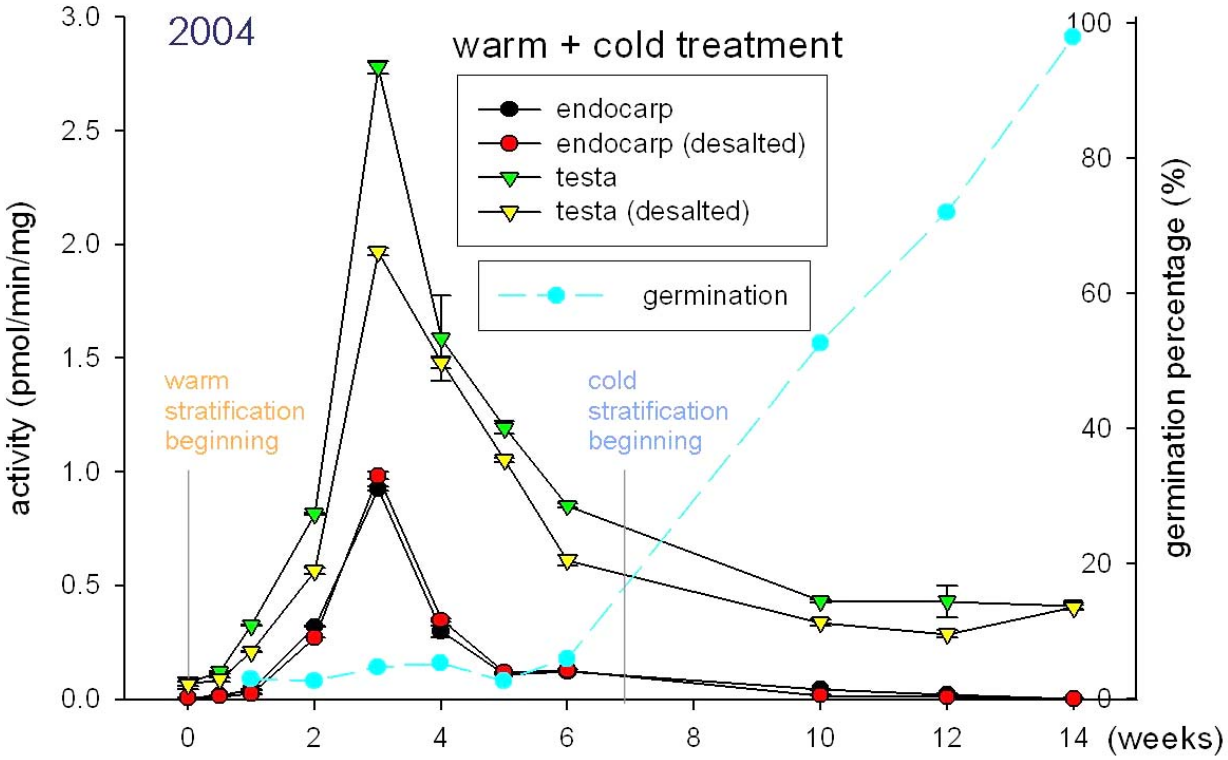


**Figure 15** *P. campanulata* sulfatases activity and percentage of germination during warm plus cold stratification.

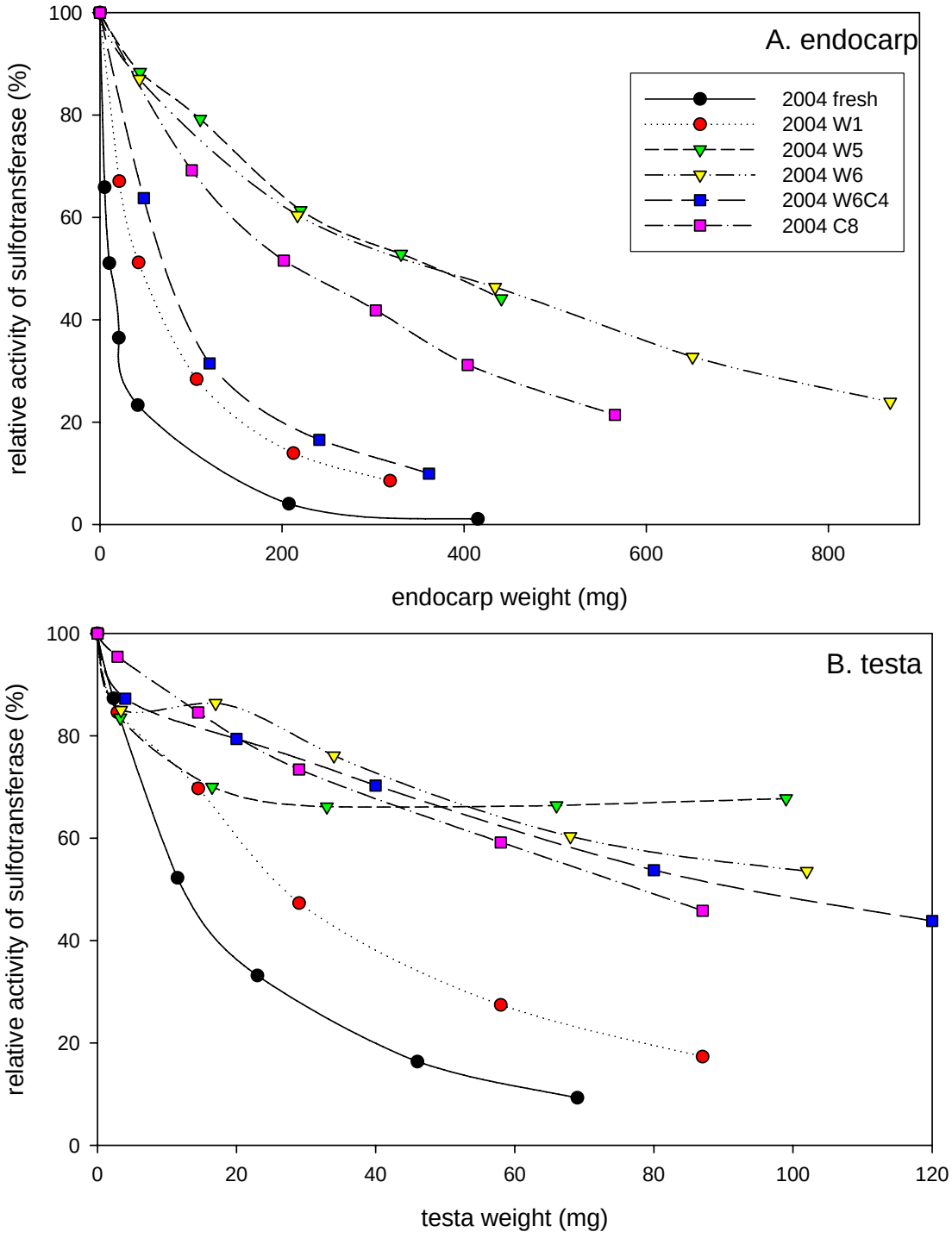


**Figure 16** The activities of phenol sulfotransferase (Rat ST1A1) were inhibited by extract from *P. campanulata* seeds during warm/cold stratification. A, inhibition of endocarp extract. B, inhibition of testa extract (Tsai YY, 2005, unpublished data).

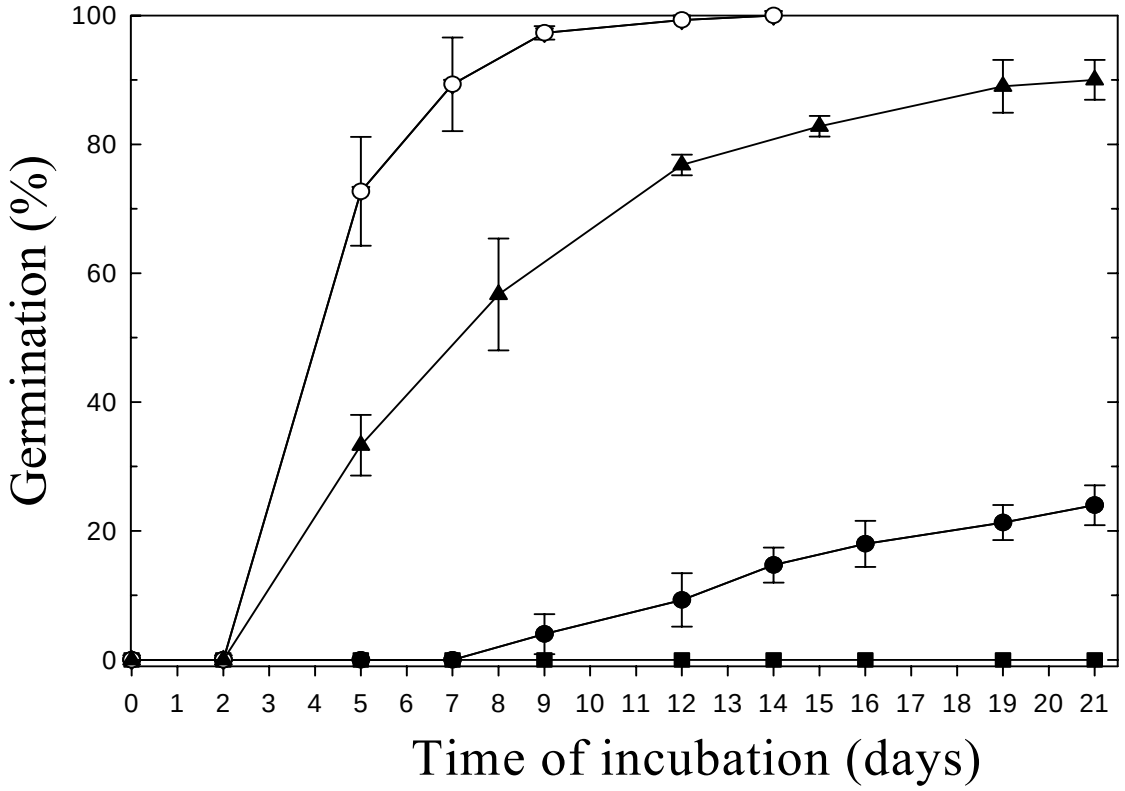


**Figure 17** Removal of testa and endocarp readily remove seed dormancy of *P. campanulata*. Germination percentage of *P. campanulata* seeds with both the endocarp and seed coat removed (open circles), with the endocarp removed (seed coat retained) (closed circles), with the endocarp removed and treated with 100 ppm $GA_3$ (closed triangles), and intact seed (closed squares) was plotted against time of incubation (Chien *et al*., 2006).

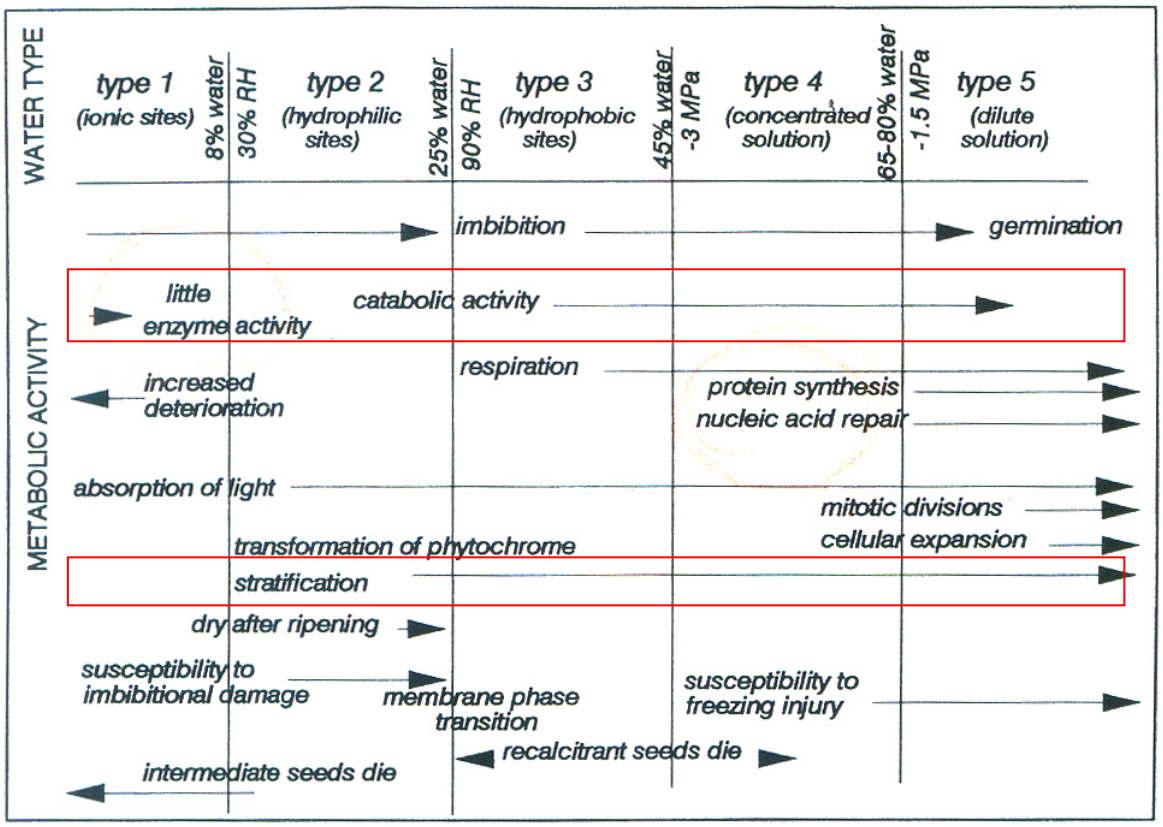


**Figure 18** The relation between water uptake and seed physiology. (Vertucci, 1993)

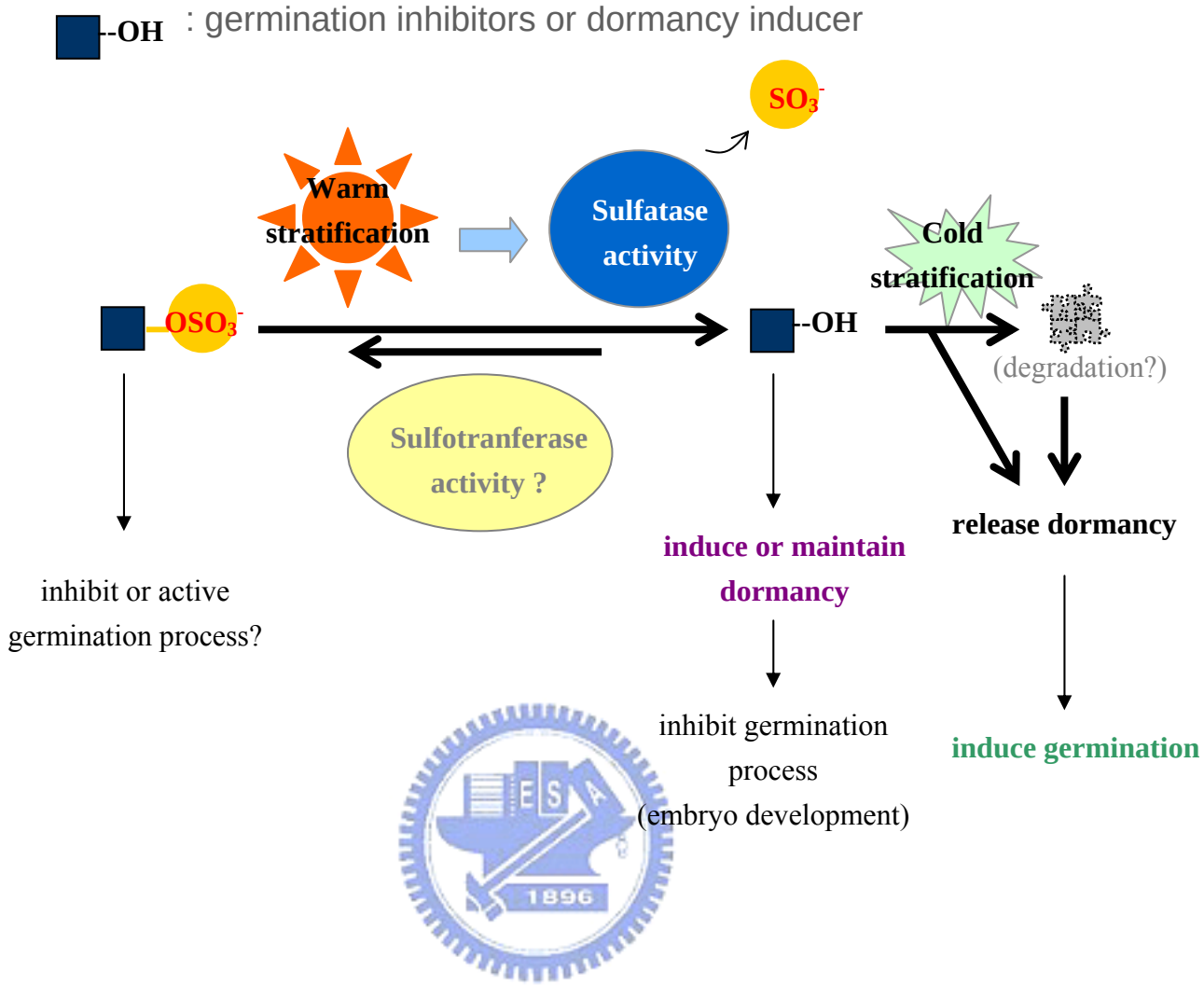


**Figure 19** The hypothesis of desulfation regulation in seed dormancy and germination. It speculated that seed testa and endocarp contain several metabolites to regulate dormancy and inhibit germination.

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# APPENDIX

## MATERIALS AND METHODS

## 藥品與方法

### A. Equipments

| equipment | Company |
|---|---|
| Hitrap desalting column | Pharmacia (Hong Kong) |
| U3300 UV/VIS Spectrophotometer | Hitachi |
| F-4500 Fluorescence Spectrophotometer | Hitachi |
| Orbital Shaker Incubator | DEHG YNG |
| Uitrafiltration System | Amicon |
| Eppendrof centrifuge 5410 | Eppendrof |
| Himac CR 22G 高速離心機 | Hitachi |
| pH meter | Fisher Scientific |
| PRO 250 homogenizer | PRO Scientific |
| Mixer Mills MM 301 | Retsch |

## B. Chemicals and Reagents

(1) Extract buffer

| reagent | final conc. | MW | others |
|---|---|---|---|
| Tris-HCl | 10mM | 121.14 | pH=7.4 or pH=8.0 |
| Sucrose | 125mM | 342.30 | |
| Glycerol | 10% | | |
| DTT (dithiothreitol) | 1mM | 154.2 | 使用前加入，加入後的 buffer 保存於 4℃。置於-20℃冰箱，拿出後放入乾燥箱備用→易吸水! |
| EDTA | 1mM | 372.24 | |

| reagent | stock conc. | MW | others |
|---|---|---|---|
| PMSF | 200mM | 174.2 | 以 isopropanol 做 solvent。<br>**buffer 在使用前皆需要加入 1mM 的 PMSF。** |

(2) The reagent of sulfatase/ST assay with fluorescence spectroscopy

| **reagent** | **Stock conc.** | **MW** | **others** |
|---|---|---|---|
| 2-mercaptoethanol ($C_2H_6OS$ ) | 250mM | 78.13 | Fluka,濃度≧99%，d=1.115，置於防爆箱。 |
| Tris-base | 0.2M | 121.14 | pH=7.0(視需求而定) |
| β-naphthol | 0.5mM | 144.2 | 怕光，置藥品櫃，分裝時用棕色的 eppendoff；acetone 置防爆櫃 |
| MUS (4-methyl-umbelliferyl sulfate) | 10mM | 294.3 | 置-20℃冰箱，取出時先置於乾燥箱。溶液需避光。 |
| MU (4-methyl-umbelliferone) ($C_{10}H_7O_3Na$ ) | 1 μM | 198.2 | 置乾燥箱，store at R.T.，黃色粉末；350nm 吸收, 450nm 發出螢光 |
| PAP (adenosine 3',5'-diphosphate) | 1mM | 427.2 | 置-20℃冰箱，取出時先置於乾燥箱。以$A_{260}$定濃度(以dd$H_2O$歸零，ε=15100 $M^{-1}cm^{-1}$) |
| AMP (adenosine -5'-monophosphate) | 1mM | 368.2 | 置-20℃冰箱，取出時先置於乾燥箱。以$A_{260}$定濃度(以dd$H_2O$歸零，ε=15400 $M^{-1}cm^{-1}$) |

C. 酵素活性計算公式

每 *mg* 種子組織萃取酵素活性　　( *pmol* / min/ *mg* )

$$= \frac{\left(\text{E}x450\text{ 改變量} \times \text{萃取液總體積}\ (mL) \times 10^{6}\right)}{\left(\varepsilon \times assay\ \text{加入的萃取液體積}\ (\mu L)\right)} \div \text{總組織重}\ (mg)$$

# AUTOGRAPHY

## Education

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## Experience

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## Publication

1. 江秉潔,邱靜玟,陳羿蓉,陳韋宏,林志生。食藥用菇類專利趨勢分析 (The Patent Analysis of Edible or Medical Fungi)。食品工業，37:5，民 94.05，頁 51-60。