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碩士論文

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Effect of Testa & Endocarp Extracts of *Prunus campanulata* Maxim on Seed Germination

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摘　　要

種子休眠是植物最重要的生理現象之一，但是涉及休眠機制的許多關鍵因子至今仍不明朗。我們利用臺灣原生植物——山櫻花種子為材料，探討種子休眠的問題。山櫻花種子呈現深度休眠的特性，研究重點是利用山櫻花種子之種皮和內果皮的萃取液，闡明種皮和內果皮所含的化學物質在山櫻花種子休眠上所扮演的角色。有許多研究報告發現胚在休眠控制上的重要性，例如阿拉伯芥、蕃茄和菸草等。但我們的研究顯示山櫻花種子胚本身不是維持休眠的主因，反而是來自種子的種皮和內果皮。藉由這些種皮和內果皮之萃取液對阿拉伯芥種子發芽和山櫻花胚生長的研究，結果證實有抑制作用，且層積處理可改變種皮和內果皮內之抑制物質對種子發芽的抑制能力。我們的研究數據顯示，種皮和內果皮這類來自母系的組織在山櫻花種子的休眠上扮演極關鍵的角色。

Effect of Testa & Endocarp Extracts of *Prunus campanulata* Maxim on Seed Germination

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ABSTRACT

Seed dormancy is one of the most pivotal physiological phenomena of the plants. But many key factors involved in the mechanism of dormancy are still not well known. The dormancy seeds of *Prunus campanulata* Maxim, a native species in Taiwan, was selected to study the mechanism of dormancy. We focus on the extracts of testa and endocarp of the seeds to elucidate the chemical roles on the seed dormancy of *P. campanulata*. There are many reports usually emphasizing the importance of embryo in controlling dormancy of plants such as *Arabidopsis*, tomato and tobacco. But our results showed that the *P. campanulata* embryo itself could not remain dormancy without intact testa and endocarp, by contrast, the growth of embryo could be repressed by the extracts of testa and endocarp from fresh *P. campanulata* seeds. Via the effect of the testa and endocarp extracts of *P. campanulata* from various stratification treatments on the germination of *Arabidopsis thaliana* seeds, we confirmed that miscellaneous stratifications can alter the repressive ability of endocarps and testae for germination. All these data showed that the *P. campanulata* testa and endocarp that are derived from the maternal original tissue play an essential role in seed dormancy.

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ABBREVIATIONS

W6	Seeds treated with 6 weeks warm stratification
C8	Seeds treated with 8 weeks cold stratification
W6C8	Seeds treated with 6 weeks then 8 weeks stratification

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Chapter 1 Introduction

1.1 The significance of dormancy

Seed dormancy can be defined as the temporary failure of a living seed to germinate under favorable conditions of germination (Simpson 1990). From the view of the evolution, the mechanism of seed dormancy is designed to survive the ominous and hostile environment. Seed dormancy is released only under the circumstances which can promote the probability of the seedling survival. (Fenner, Thompson. 2005) For dormant seeds, when the condition is suitable for germination but not for the development of seedling, the seeds keep the dormancy going on. For example, when the water and nutrition conditions can meet the requirement of germination of the non-dormant seeds or the dormancy-breaking seeds, the seeds germinate. But the same conditions of water and nutrition may not urge the dormant seeds of the same species to germinate unless the dormancy is released or broken. How can the plant seeds predict if the situations are suitable for their development? The adaptation of seeds to the variety of environment is attributed to the mechanism of evolution. The dormancy is one of the most successful ways of adaptation.

1.2 The relation between dormancy/germination and ABA/GA

There were many researches to focus on the relationship of abscisic acid (ABA), or Gibberellic acid (GA) with dormancy. Some showed that ABA synthesis in the embryo and endosperm contributes to the induction of seed dormancy. (Lefebvre *et al*., 2006) Increased ABA biosynthesis is associated with the dormant state (Cadman *et al*., 2006). But in *Prunus campanulata* Maxim, the covering layers can impose the dormancy. A decrease of ABA content of the covering layers and an increase of embryonic GA_4 content go along with dormancy-break. (Chen *et al.*, 2007) Whereas some studies using the methods of grafting or reciprocal crosses between ABA

mutation and wild type in *Arabidopsis*, tomato, *Nicotiana* elucidated that only ABA produced by the embryo itself, but not maternal ABA, was necessary to induce and impose a lasting dormancy. (Frey *et al.*, 2004) The maternal ABA in seeds is produced by the seed-covering layers as well as the leaves or roots of the plant. The maternal ABA is synthesized by the maternal vegetative tissues mainly from the roots and the leaves. (Sauter *et al.*, 2001) ABA is then transported through the xylem and the phloem to the seed. ABA recirculation and redistribution is a significant component of the root-to-shoot signaling process. It often results form pH changes. (Nambara *et al*., 2003) The ABA produced by roots can sink leaves, fruits and seeds through the xylem, so as the ABA produced by the leaves sink roots, fruit and seed through the phloem. The vascular tissue of roots, hypocotyls and leaves is an important site of ABA biosynthesis in vegetative tissues (Koiwai *et al*., 2004) However, the maternal ABA can affect the seed development. At least in *Arabidopsis*, the maternal ABA of the seed is involved in preventing viviparous germination. (Raz *et al*., 2001) Vivipary is a normal phenomenon in *Rhizophora mangle* (the mangrove). (Kermode, 2005) But vivipary is an abnormal phenomenon in many plants such as a rice or *Arabidopsis*. (Gubler *et al*., 2005) In *Arabidopsis*, the regulation of vivipary requires two processes, the first stage is regulated by the *FUS/LES* genes and the second stage requires ABA. (Raz *et al*., 2001) In addition to the maternal original ABA in the seed, ABA can also be synthesized by the embryo and the endosperm. (Lefebvre *et al*., 2006)

ABA in the seed can play roles of assisting the seed development, keeping the seed in the state of dormancy, desiccation tolerance, reserve accumulation, pigment synthesis such as anthocyanin or flavonoid in the seed coat and seed capsule maturation. A class of flavonoids esterified with sulfates groups are of common occurrence in a number of plant families, especially in the *Asteraceae*. (Varin *et al*.,

1992) Anthocyanins and flavinol which is a subgroup of the flavonoid pigments may have a primary role in protecting against active oxygen species during growth and stress. (Yamasaki H., 1997, Rice-evans *et al*., 1997)

1.3 The relation between dormancy and cell division cycle

Seed dormancy processes at least two sequential stages: first, the embryo growth of a seed is arrested because of cell division being suspended, second, the embryo kept in dormancy. ABA can promote the expression of a cyclin-dependent kinase inhibitor (ICK1) which would conduct the cell cycle arrested at the G1/S transition.(Wang *et al*., 1998) The endogenous ABA from the embryo is required for arrest in G1.(Finkelstein *et al*., 2002)

1.4 The relation between dormancy-breaking and stratification

Cold stratification (chilled process) can break dormancy and promote germination. In the presence of light, the helix-loop-helix transcription factor SPAULA(SPT) can regulate the cold response, loss of SPT allows germination without stratification. SPT is a repressor of *GA3ox*(the gibberellin biosynthetic genes GA3 oxidase). (Penfield *et al*., 2005)

In the research of *A. thaliana* Cvi , Sonia *et al.* (2004) found that the chemical compounds or treatments that induce the germination of dormancy seed are, in increasing order of efficiency: GAs (GA_3,GA_4,GA_7) << NO_3^- < fluridone < GA_3 + fluridone < 13℃ sowing < 4 days chilling. Fluridone is the inhibitor of the synthesis of ABA.

1.5 The genes of ABA

From the view of the gene expression, ABA 8'-hydroxylase is the key

catabolic enzyme for ABA. The CYP707A gene family which is constituted of four members (CYP707A1 to CYP707A4) encodes ABA 8'-hydroxylase in *Arabidopsis thaliana*. (Okamoto *et al*., 2006) The reduction of ABA levels in mid-maturation is contributed by the expression of CYP707A1 that located in the vascualar tissue in the embyro, whereas ABA reducing from late-maturation to germination is contributed by the expression of CYP707A2 gene that located in both embryo and endosperm. CYP707A1 and CYP707A3 also contribute to ABA catabolism during postgermination growth, while the expression of CYP707A4 was found only in the silique envelopes. (Kushiro *et al*., 2004)

1.6 The character of *P. campanulata* Maxim

P. campanulata Maxim is a local species plant of Taiwan. (Figure 1) It shows a strong dormancy. In the study of Chien CT *et al*.(2002), they showed that a combination of warm and cold stratifications could break dormancy and elevated the percentage of germination of *P. campanulata.* We used *P. campanulata* for studying the mechanism of dormancy. To explore the relationship between stratification and dormancy as well as the relationship among embryo, endocarp and testa, we utilized the endocarp and testa extracts of *P. campanulata* with various stratification treatments to test their effect on the germination of *A. thaliana* seeds.

1.7 The classification of *P. campanulata*

From the view of the anatomy, the embryo with cotyledons is warped by the endosperm which is coated by the testa the seed coat. Then the seed of *P. campanulata* is enveloped by the endocarp. (Figure 2, 3) Both the testae and the endocarps of *P. campanulata* are maternal tissues.

The testa derives from the integuments of the ovule, while the endocarp derives from the innermost section of the ovary. (Nambara *et al*., 2003)

From the view of the classification.

The following is the classification of *Prunus campanulata* Maxim:

Kingdom：Plantae

Division：Spermatophyta

Class：Angiosperma

Order：Rosale

Family：Rosaceae

Chapter 2 Materials and Methods

2.1 Materials

The seeds of *Prunus campanulata* Maxim were harvested from 2000 meter elevation of Ali Mountain in Chiayi County, central Taiwan during May in 2006 and 2007. Then the seeds were saved in cooling requirements at 4℃ to keep them fresh, alive and be at our disposal. By fresh seeds we mean that the seeds didn't process any stratifications.

2.2 The warm stratification processions.

The Method of the warm stratification was based on the study of Chien CT *et al*.(2002). Fresh *P. campanulata* seeds were intermingled with vaporous sphagnum in a polyethylene bag capable of sealing. In the warm stratification conditions, the sealed polyethylene bag which contained the seeds and moist sphagnum in a desired period were kept alternative between at 30℃ in the fluorescent light at the lumen of 80-100 μE/m^2/s for 12 hours and 20℃ in the dark for 12 hours. The bags were opened for checking moisture one time a week.

2.3 The cold stratification processions.

The Method of the cold stratification was based on the study of Chien CT *et al*.(2002). In the cold stratification conditions, the sealed polyethylene bag which contained the seeds and moist sphagnum in a desired period were kept at 4℃ in the dark. The bags were opened for checking moisture one time a week.

2.4 The test of the germination of *P. campanulata* in alternative stratifications.

The germination testing of *P. campanulata* in alternative stratifications was processed in the incubating box kept the temperature alternative between 30℃ in the

fluorescent light at the lumen of 80-100 μE/m^2/s for 12 hours and 20℃ in the dark for 12 hours in the period of 12 weeks.

2.5 The preparation of the testa extracts and germination test

The testae were removed from the 300 seeds of *P. campanulata*. Then the testae which were added to 5ml ddH_2O were ground to a glue-looking state in a grinding machine.

The grinding frequency was 25 times per second in 30 seconds of operation interval. Next, take a rest for one minute. Repeat the same grinding procedure four times. The ground testae were sent to the high temperature at 121℃ for 30 minutes for eradicating bacteria and molds. At the same the high temperature destroyed proteins. The water which was added to the testae would vaporize off in the high temperature. When the testae cooled down, methanol of 80% concentration were added to them. The proportion was 20ml methanol to testae of 300 seeds in the condition for testing the germination of *Arabidopsis* seeds. But for testing the germination of *P. campanulata* seeds the proportion was changed to 35ml methanol to testae of 300 seeds. The chemicals in the testae can dissolve in methanol gradually. The solvent was kept at 4℃ for 24 hours. Afterwards the solvent was stirred and centrifuged at 17800 rpm for 30 minutes. The upper clean liquids were collected. That was the testa extracts dissolved in methanol. Then the extracts can be dropped to the filter papers in Petri dishes. In the case of testing the germination of *Arabidopsis* seeds, a filter paper was set in every Petri that was dropped 2 ml extracts. But in the case of testing the germination of *P. campanulata* seeds, a Petri dish contains two filter papers into which 4ml extracts were dropped. The seeds cannot be sowed on the filter papers until the solvent vaporized completely and the filter papers dried off. After the filter papers were desiccated, the filter papers were moistened by water in order to preparing for

the imbibition of the seeds. 1.5ml water was added to per Petri dish in the case of testing the germination of *Arabidopsis* seeds, whereas 4ml water was added to per Petri dish in the case of testing the germination of *P. campanulata* seeds. Then the seeds concerned were sowed on the filter papers. (Flow Chat 3)

2.6 The preparation of endocarp extracts and germination test

The endocarps were removed from the 300 seeds of *P. campanulata*. (300 is the quantity for 3 repetitions.) Then the endocarps were ground to a powder-looking state in a grinding machine.

The grinding frequency was 25 times per second in 30 seconds of operation interval. Next, take a rest for one minute. Repeat the same grinding procedure four times. The ground endocarps were sent to the high temperature at 121℃ for 30 minutes for vanquishing bacteria and molds. At the same the high temperature destroyed proteins. When the endocarps cooled down, methanol of 80% concentration were added to them. The proportion was 40ml methanol to endocarps of 300 seeds in the condition for testing the germination of *Arabidopsis* seeds. But for testing the germination of *P. campanulata* seeds the proportion was changed to 70ml methanol to endocarps of 300 seeds. The chemicals in the endocarps can dissolve in methanol gradually. The solvent was kept at 4℃ for 24 hours. Afterwards the solvent was stirred and centrifuged at 17800 rpm for 30 minutes. The upper clean liquids were collected. That was the endocarps extracts dissolved in methanol. Then the extracts can be dropped into the filter papers in Petri dishes. In the case of testing the germination of *Arabidopsis* seeds, a filter paper was set in every Petri into which 2 ml extracts were dropped. But in the case of testing the germination of *P. campanulata* seeds, a Petri dish contains two filter papers into which 4ml extracts were dropped. The seeds cannot be sowed on the filter papers until the solvent vaporized completely and the filter papers dried off.

After the filter papers were desiccated, the filter papers were moistened by water in order to preparing for the imbibition of the seeds. 1.5ml water was used to per Petri dish in the case of testing the germination of *Arabidopsis* seeds, whereas 4ml water was used to per Petri dish in the case of testing the germination of *P. campanulata* seeds. Then the seeds concerned were sowed on the filter papers.

2.7 The favorite growth condition for *P. campanulata* and *A. thaliana.*

The *P. campanulata* seeds were incubated in the environment at the temperature alternative between 30℃ in the fluorescent light at the lumen of 80-100 μ E/m^2/s for 12 hours and 20℃ in the dark for 12 hours, whereas the *A. thaliana* seeds incubated in the environment at the temperature alternative between 22℃ in the fluorescent light at the lumen of 80-100 μ E/m^2/s for 12 hours and 18℃ in the dark for 12 hours.

2.8 The imbibition of seeds

Both of the warm stratification and chilling stratification of the *P. campanulata* seeds were moistened. The imbibition of seeds before the germination is one of the requirements for germination.

2.9 The test of germination of *P. campanulata* embryo without the testa and endocarp

The endocarp and testa were removed from the *P. campanulata* seed. The cotyledons were cut down a half in order to favor the radicles of *P. campanulata* seeds to contact the liquids of a filter paper in a Petri dish. (Figure 14 ; Flow Chat 2)

Chapter 3 Results

3.1 The dormancy-breaking and germination of *Prunus campanulata* Maxim in various stratification

For the intact *Prunus campanulata* Maxim seeds with endocarps under the auspices of the stratification first of six weeks warm at 20/30℃ and then of eight weeks chilling at 4℃ , the germination percentage can culminate in nearly 100%. (Figure 6) The stratification of either the warm or the chilling alone cannot completely break dormancy, although the chilling stratification effect is greater than the warm stratification. The germination percentage of C8 *Prunus campanulata* seeds was 52%, whereas that of W6 seeds was 3.3%. But the effect of the combination of W6 and C8 stratifications on the germination of *Prunus campanulata* seeds was over the sum of the germination percentage of the individual warm and cold stratification.

3.2

When taking away the endocarps and testae with endosperm from mature seeds, the embryos can germinate in a few days in the Petri dishes with filter papers which were added water to at the temperature alternative between 30℃ in the fluorescent light at the lumen of 80-100 μE/m2/s for 12 hours and 20℃ in the dark for 12 hours. (Figure 4, 5) The mature seeds without endocarps were cut into two halves in the cross direction, the embryos which contact the endosperms with testae can also germinate in a few days in the Petri dishes with filter papers which were added water to at the temperature alternative between 30℃ in the fluorescent light at the lumen of 80-100 μE/m^2/s for 12 hours and 20℃ in the dark for 12 hours. In contrast, it took a few months to complete the germination for the whole seed.

3.3 The effect of *P. campanulata* endocarp and testa extracts on the germination of *Arabidopsis*

The extracts of fresh *P. campanulata* endocarps contain some chemicals that can repress nearly completely the germination of wild type *Arabidopsis thaliana*. (Figure 7,8,9,10,11,12,13) The repressive ability of W6 endocarp extracts was reduced, whereas that of C8 endocarp extracts was still great for the germination of *A. thaliana* seeds. The effect of testa extracts on *A. thaliana* was less than that of endocarp extracts.(Table 1,2,3)

3.4 The effect of *P. campanulata* endocarp and testa extracts on the growth of the embryos of *Prunus campanulata* Maxim seeds which were removed testae and endocarps

Both of testa and endocarp extracts can represse the growth of radical of the embryo partially.

Chapter 4 Discussion

4.1 The germination of *P. campanulata* Maxim seeds with/without endocarp and testa

It has been elucidated that only ABA produced by the embryo itself, but not maternal ABA, is necessary to induce and impose a lasting dormancy in *Arabidopsis* and *Nicotiana plumbaginifolia* (tobacco). (Karssen *et al*., 1983 , Frey *et al.*, 2004) But our research showed that the embryo of *P. campanulata* seeds cannot conduct the maintenance of dormancy when the endocarps and testae were taken away. In the situation of removing endocarp from *P. campanulata* seed, the embryo can germinate even endosperm and testa still contact the embryo in the condition of testa and cotyledons cut down a half.(Figure 4) While in the full seed state by which we mean the embryo covered by testa but without endocarp, the testa and endosperm is kept intact in the *P. campanulata* seeds, the germination of the embryo can be arrested. It is apparent that the maintenance of dormancy of *P. campanulata* seeds should not be contributed alone to the the embryo.

4.2 The interaction between *Arabidopsis thaliana* seeds and extracts from *P. campanulata* seeds

The influence of *P. campanulata* on other plants, we chose *Arabidopsis thaliana* seeds. According to the experimental results, the endocarps of fresh *P. campanulata* seeds containing some chemical (or chemicals) can repress the germination of *Arabidopsis* seeds. To subtract the noise background of mold, it is apparent the endocarps of fresh *P. campanulata* seeds have more powerful repression than the testa of fresh *P. campanulata* seeds did. (Figure 7,11) In fact the fresh *P. campanulata* seeds are in the state of deep dormancy. There must be some proteins and chemicals taking the control of dormancy and repressing the germination of fresh *P.*

campanulata seeds. From our research, the endocarps of *P. campanulata* seeds may play a role to keep seeds in the state of dormancy or repress the germination of seeds. The factors involved in dormancy are not only physical but also chemical.

4.3 The effect of extracts from various stratification on the germination of *A. thaliana* seeds

Compared with the endocarp treated by stratification process of six weeks warm and eight weeks cold, the fresh endocarp still had more repressive ability to control the germination of *A. thaliana* seeds. The inhibit ability of endocarp extracts in order of efficiency was

W6C8 Endocarp< W6 Endocarp < C8 Endocarp < Fresh Endocarp (Figure 12; Table 1)

This result makes sure that the chemicals of the endocarps of *P. campanulata* seeds are affected by temperature changes. The stratification process can increase the percentage of germination. The results of our research confirm that the stratification process can release the repressive ability of the endocarp of *P. campanulata* seeds.

4.4 The significance of the warm stratification

The endocarp extracts of W6 stratification had less repressive ability to the germination of *A. thaliana* seeds. In the case, the germination of *A. thaliana* seeds was nearly 42.7%. The supposed chemicals which can repress germination seemed decayed in the process of warm stratification. But the test germination of the whole *P. campanulata* seeds which were processed with W6 stratification, their germination percentage was still 3.3% near to the situation of fresh seeds. According to the study of Chen *et al*. (2007), they found that the ABA content in endocarps and testae decreased after stratification, and the ABA content with warm stratification was less

than that with cold stratification. The order of ABA content was W6C8 Endocarp< W6 Endocarp < C12 Endocarp < Fresh Endocarp. The research result of Chen *et al*. in term of ABA agrees with our result in term of germination of *A. thaliana* seeds affected by endocarp extracts. It is obvious that the repressive chemicals leached from endocarps and testae during the stratification, especially the warm stratification. On the other hand, the warm stratification could promote the catabolism of repressive chemicals such as ABA. But germination of *P. campanulata* seeds with warm stratification was still so insignificant. Why? The embryos of *P. campanulata* seeds seemed still under arrest after warm stratification. It was possible that the restriction was released but the promoting factors which could induce the growth of embryo had not been produced yet.

4.5 The significance of the cold stratification

The endocarp extracts of cold 8 weeks (C8) stratification still have very greatly repressive ability to the germination of *A. thaliana* seeds. In the case, the germination of *A. thaliana* seeds was nearly 10%. But the test germination of the whole *P. campanulata* seeds which were processed with C8 stratification, their germination percentage was over 50%. In fact, the C8 seeds must be transposed from 4℃ to 30℃/20℃ for testing germination. Cold stratification seemed to promote the producing of growth factors maybe something such as GA. Then at the 30℃/20℃ environments which were the same as the condition of warm stratification the repressive chemicals decayed gradually, the promoting chemicals commenced the effect. In *Arabidopsis*, cold stratification at 4℃ can promote and synchronize seed germination because of inducing the *GA3ox1* mRNA expression in the entire radicle and increasing the product of GA. (Yamauchi *et al.*, 2004) In *P. campanulata*, we suggest that the cold stratification can expedite the synthesis of growth factors for germination.

4.6 The hypothesis of stratifications

The repressive chemicals of testa and endocarp extracts of *P. campanulata* seeds may be ABA, but it is necessary to be confirmed. But from our study, we can confirm that *P. campanulata* seeds must process two stages for dormancy break. First, take off the impediment with the warm stratification, afterwards, promote the growth with the cold stratification. The embryo which was removed the endocarp and testa could germinate without stratification. The embryo itself of *P. campanulata* is ready for germinating. As a consequence, we can revise the previous hypothesis that the cold stratification can promote the growth factors something like GA to a new hypothesis that the cold stratification can repress the factors which restrain the synthesis of growth factors.

4.7 The role of endosperm in germination

The endosperm of immature *P. campanulata* Maxim seeds is thicker than that of mature seeds. The thick endosperms can be the obstacle to the growth of radicles of embryos.(Müller *et al*. 2006) When the endosperm of *P. campanulata* was cut, the radicle could release from impediment and grow similar to germination. It is necessary to weaken the endosperm in the procession of germination. In *Arabidopsis*, ABA can inhibit endosperm rupture but dose not appreciably affect testa rupture of after-ripened seeds.(Müller *et al*. 2006) But in *P. campanulata*, the interaction between the endosperms and the chemicals which the testae or endocarps contained is not confirmed. The chemicals of endocarps or testae may affect the rupture of endosperm then to control the germination and dormancy.

Chapter 5 Conclusion

5.1

Whole seeds of *P. campanulata* show deep dormancy, whereas the embryos without testae and endocarps show non-dormancy. The maintenance of dormancy contributes to the interaction among the embryo, endosperm, testa and endocarp. The testae and endocarps have chemical as well as physical function. The concentration of chemicals in testae and endocarps will vary with the stratification. The stratifications which switch seed dormancy off may depend on the chemicals of testae and endocarps to deliver the message to the embryos.

5.2

To insight the mechanism of dormancy must integrate various views of chemicals, anatomy, physiology, histology, embryology, genomics and proteomics. The *P. campanulata* is an ideal plant for studying dormancy. But the genome of *P. campanulata* is not available in the present time. If the difficulty of getting genome data can be broken through, the mechanism of dormancy of *P. campanulata* as well as many other plants must be explicit and unequivocal.

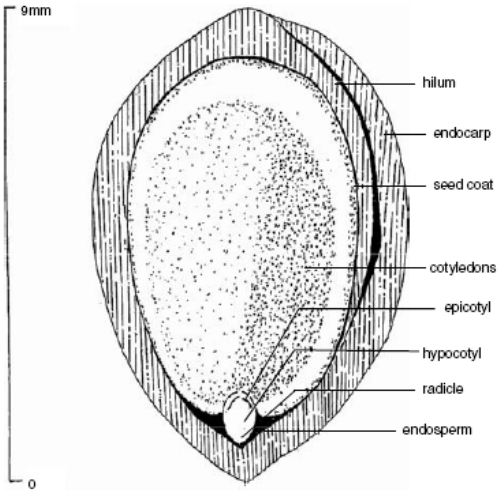
Figure 1.

Prunus campanulata Maxim can produce pink red, campanulate flowers in the spring (about from January to March).

Figure 2.

The seed of *P. campanulata* is covered by an endocarp.

Figure 3. (Courtesy of Chen SY *et al*., 2007)



The inner structure of *P. campanulata* seed

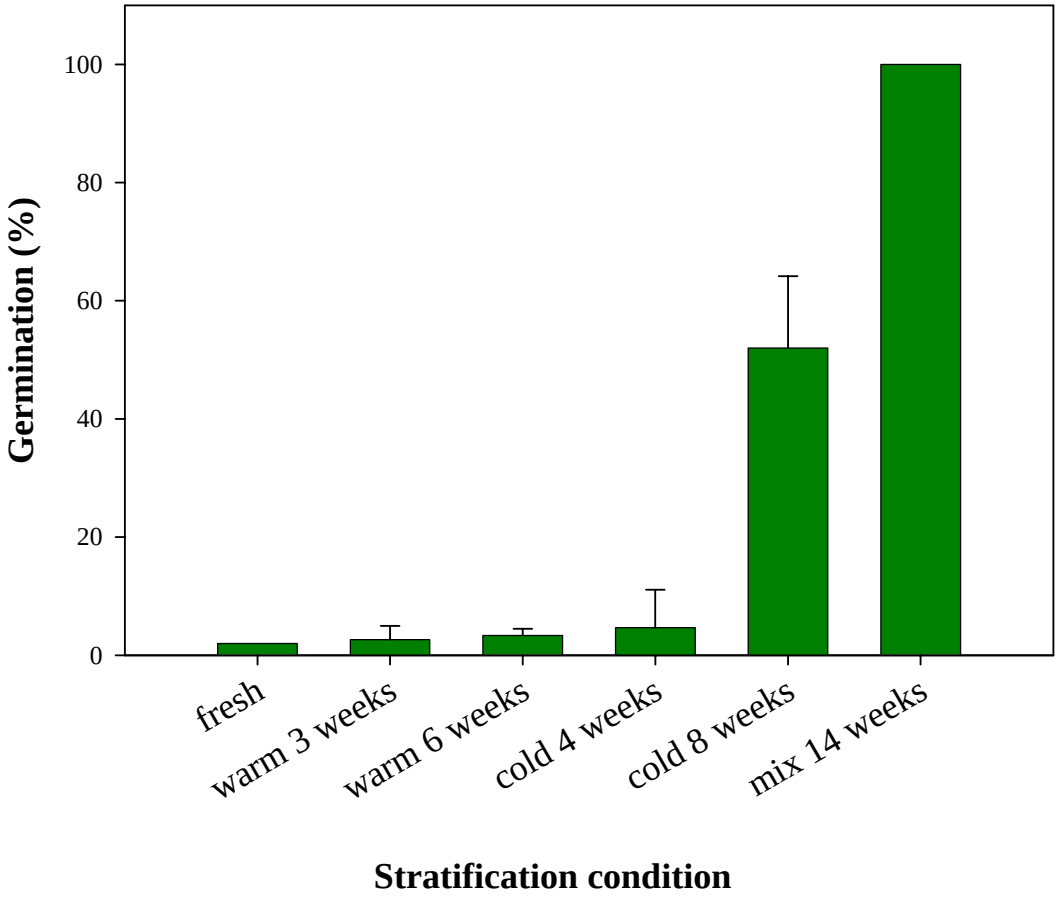
Figure 4.

Take off the endocarp, there is the testa.
Under the testa, there is the embryo which contains the radicle, cotyledons and endosperm and so on.

Figure 5.

The mature seeds from which the endocarps were token away were cut into two halves in the cross direction, the embryo which contact the endosperm with testa can germinate without stratification in a few days. In sight, it was ten days after sowing.

Figure 6. The germination test of *P. campanulata* seeds from various stratification



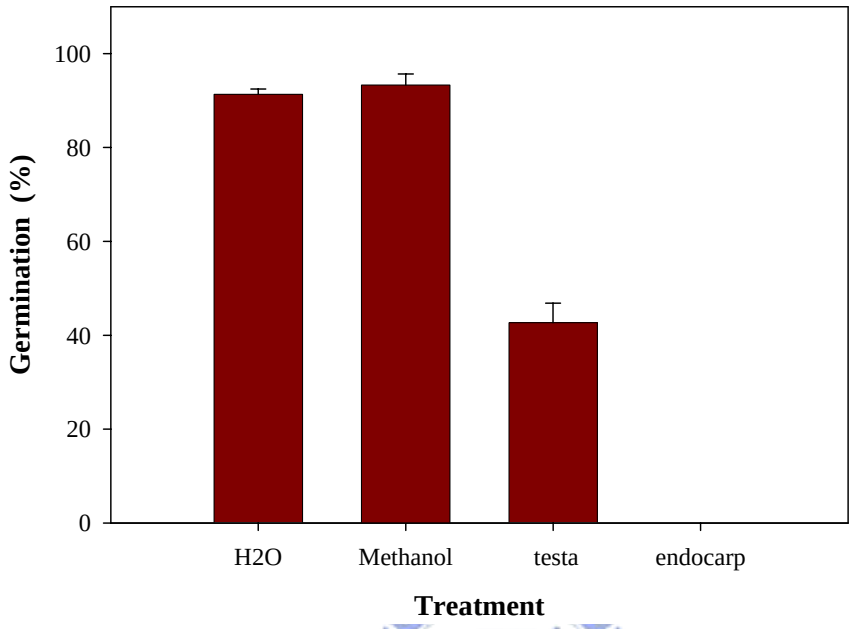
The *P. campanulata* seeds which had already been treated by various stratifications were carried out the test of germination.

The *P. campanulata* seeds were harvested in 2006.

‘ mix 14 weeks’ stands for warm 6 weeks then cold 8 weeks stratification.

The germination percentage of ‘mix 14 weeks’ seeds was nearly 100%.

Figure 7. The effect of fresh *P. campanulata* seed extracts on the germination of wild type *A. thaliana* seeds

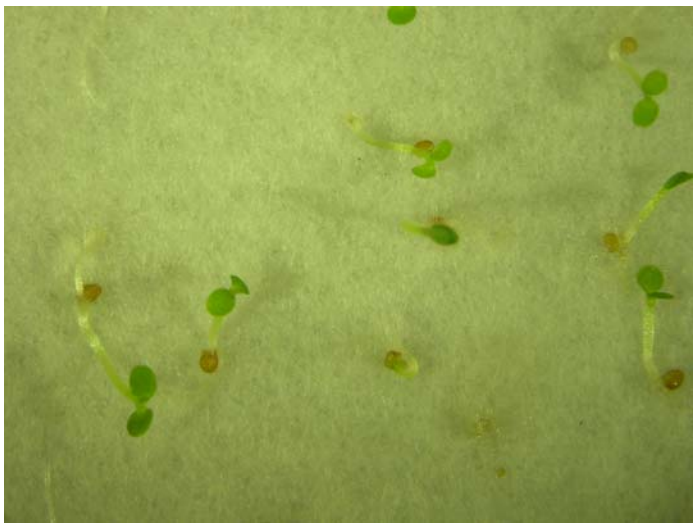


The test was processed with extracts of 100 seeds of *P. campanulata* per Petri dish for 120 hours.

50 *A. thaliana* seeds were sowed on the filter paper which contained the control or experimental materials and incubated for 120 hours.

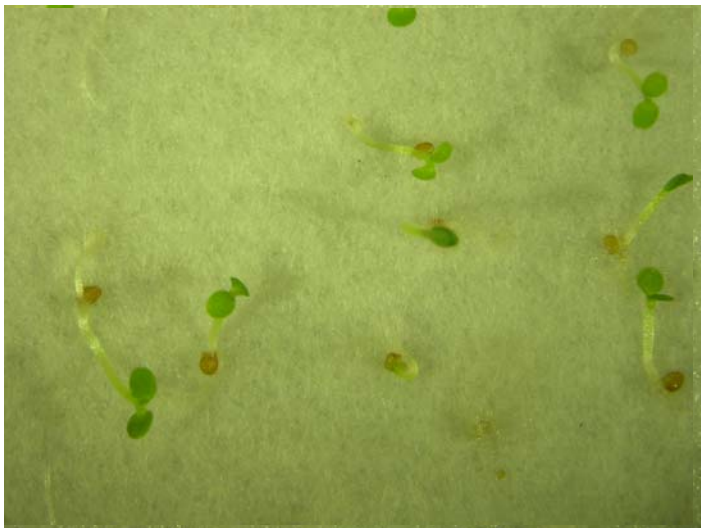
The endocarp extracts of *P. campanulata* seeds can inhibit efficiently the germination of *A. thaliana* seeds.

Figure 8. (Continued with Figure 7) The control set of the test of the effect of fresh *P. campanulata* seed extracts on the germination of wild type *A. thaliana* seeds



The *A. thaliana* seeds were sowed on the filter paper which contained water only.

Figure 9. (Continued with Figure 7) The control set of the test of the effect of fresh *P. campanulata* seed extracts on the germination of wild type *A. thaliana* seeds

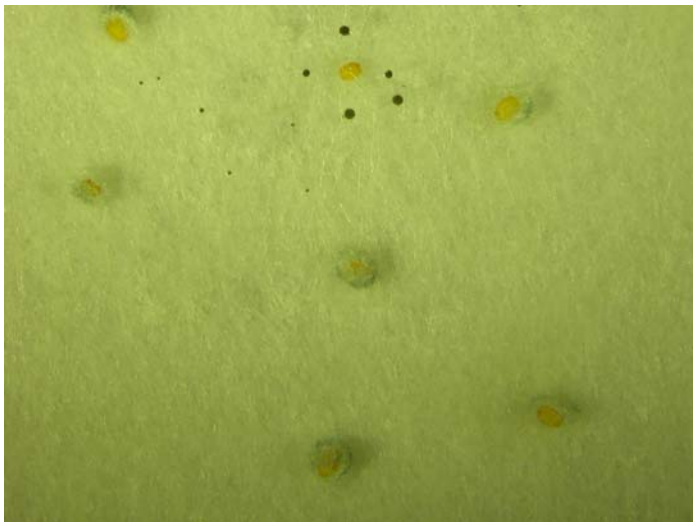


The *A. thaliana* seeds were sowed on the filter paper into which methanol was added first and dried off ,then water was added .

figure 10. (Continued with Figure 7) The experimental set of the test of the effect of fresh *P. campanulata* seed extracts on the germination of wild type *A. thaliana* seeds

The A. thaliana seeds were sowed on the filter paper which contained fresh testa extracts of *P. campanulata* seeds.

figure 11. (Continued with Figure 7) The experimental set of the test of the effect of fresh *P. campanulata* seed extracts on the germination of wild type *A. thaliana* seeds



The A. thaliana seeds were sowed on the filter paper which contained fresh endocarp extracts of *P. campanulata* seeds.
The germination of *A. thaliana* seeds were inhibited efficiently by endocarp extracts of *P. campanulata* seeds.

Figure 12. The effect of endocarp extract from different stratification on germination of *A. thaliana* seeds

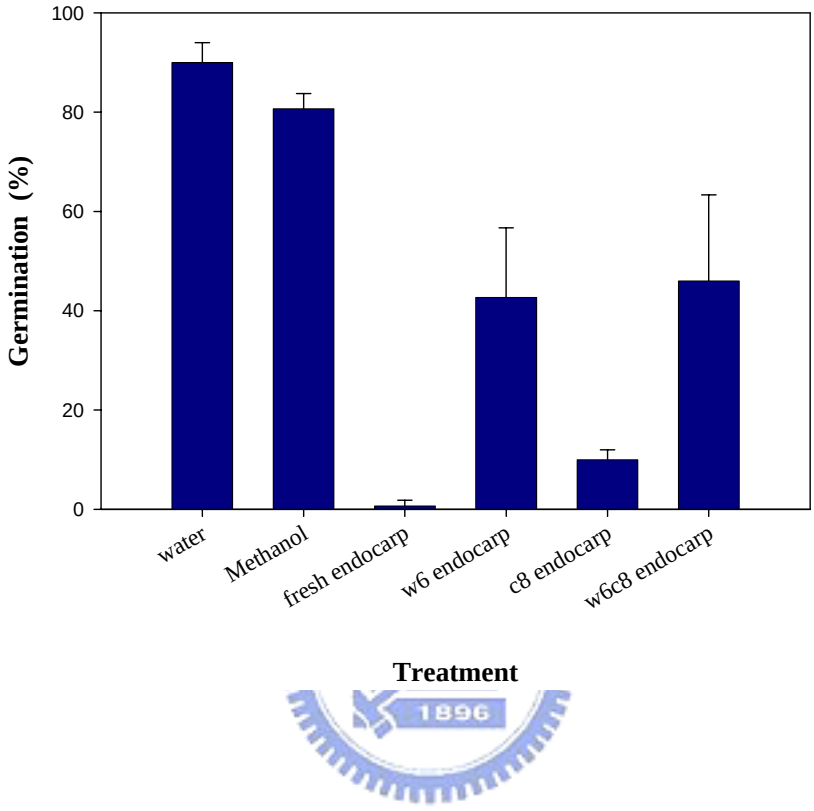


Figure 13. The effect of testa extract from different stratification on germination of *A. thaliana* seeds

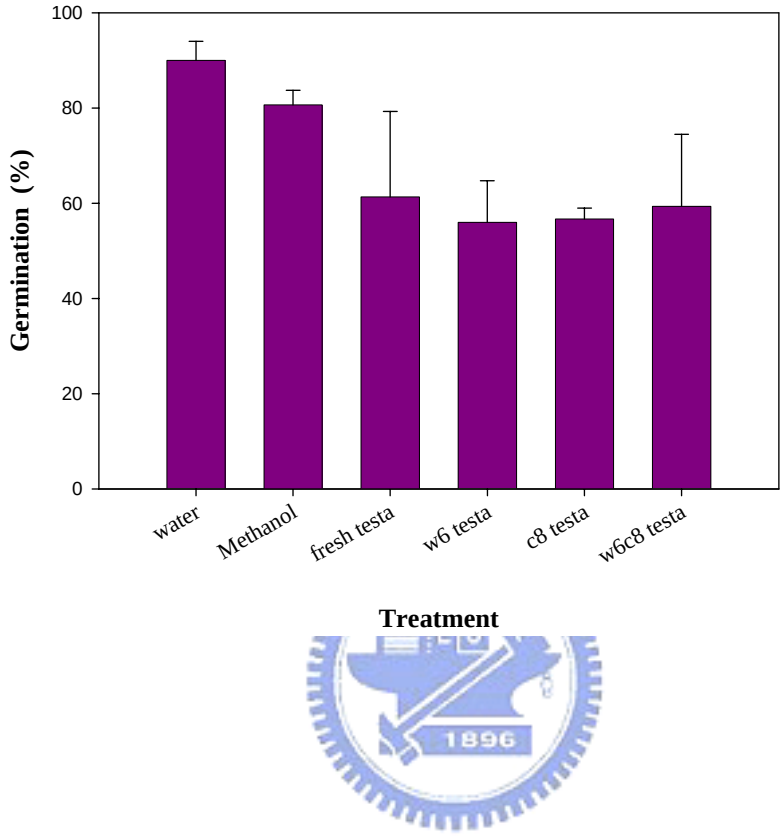
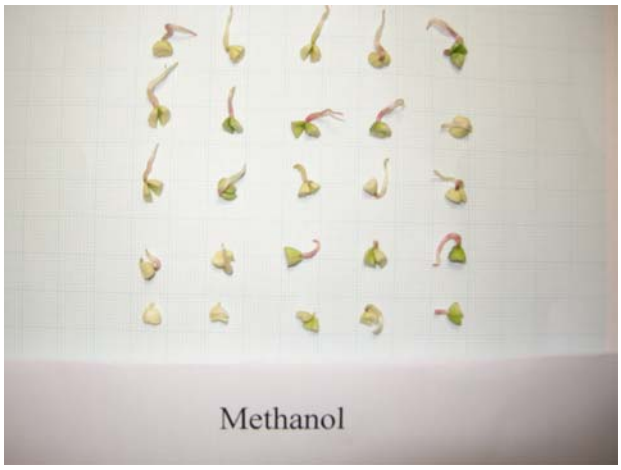
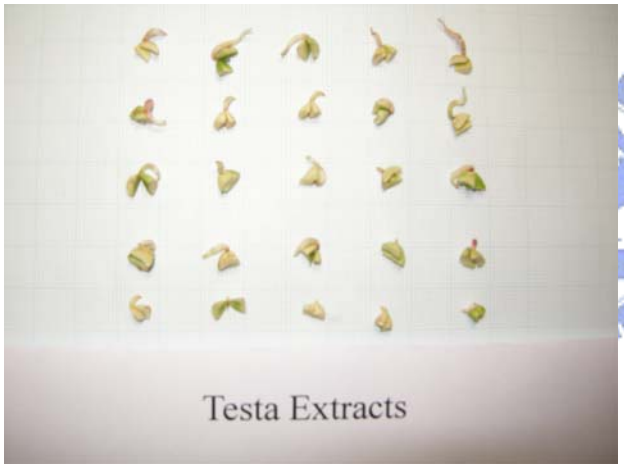


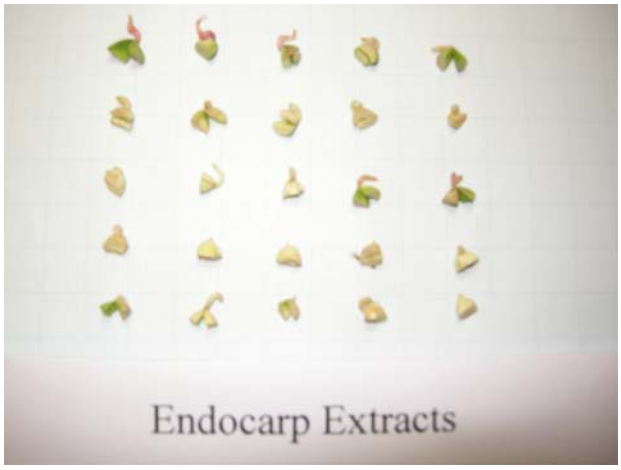
Figure 14. The effect of extracts from fresh *P. campanulata* seeds on germination of *P. campanulata* embryo which was taken off endocarp and testa with cotyledons cut down to a half.



The control set with methanol process.



The experimental set of testa extracts.



The experimental set of endocarp extracts.

Table 1. The effect of endocarp extract of *P. campanulata* seeds from different stratification on germination of *A. thaliana* seeds

Endocarp

Treatment	Germination (%)	Testa rupture (%)	Intact seedcoat (%)
Water	90.0 ± 4.0	4.7 ± 1.2	5.3 ± 3.0
Methanol	80.7 ± 3.0	6.7 ± 1.2	12.7 ± 2.3
Fresh	**0.7 ± 1.2**	**38.7 ± 6.8**	**60.7 ± 11.0**
Warm 6 weeks	**42.7 ± 14.0**	**36.7 ± 11.7**	**20.6 ± 4.2**
Cold 8 weeks	**10.0 ± 2.0**	**46.0 ± 2.0**	**44.0 ± 0.0**
Warm 6 and cold 8 weeks	**46.0 ± 17.3**	**22.7 ± 3.0**	**31.3 ± 14.5**

The tests in bold font are shown to be statistically significant through z-test with α=0.01.

Table 2. The effect of testa extract of *P. campanulata* seeds from different stratification on germination of *A. thaliana* seeds

testa

Treatment	Germination (%)	Testa rupture (%)	Intact seedcoat (%)
Water	90.0 ± 4.0	4.7 ± 1.2	5.3 ± 3.0
Methanol	80.7 ± 3.0	6.7 ± 1.2	12.7 ± 2.3
Fresh	**61.3 ± 17.9**	10.7 ± 6.1	**28.0 ± 12.5**
Warm 6 weeks	**56.0 ± 8.7**	10.0 ± 3.5	**34.0 ± 8.0**
Cold 8 weeks	**56.6 ± 2.3**	11.3 ± 5.0	**32.7 ± 4.6**
Warm 6 and cold 8 weeks	**59.3 ± 15.1**	9.3 ± 4.2	**31.3 ± 11.0**

The tests in bold font are shown to be statistically significant through z-test with α=0.01.

Table 3. The comparison between effect of endocarp and testa extracts of *P. campanulata* seeds from different stratification on germination of *A. thaliana* seeds

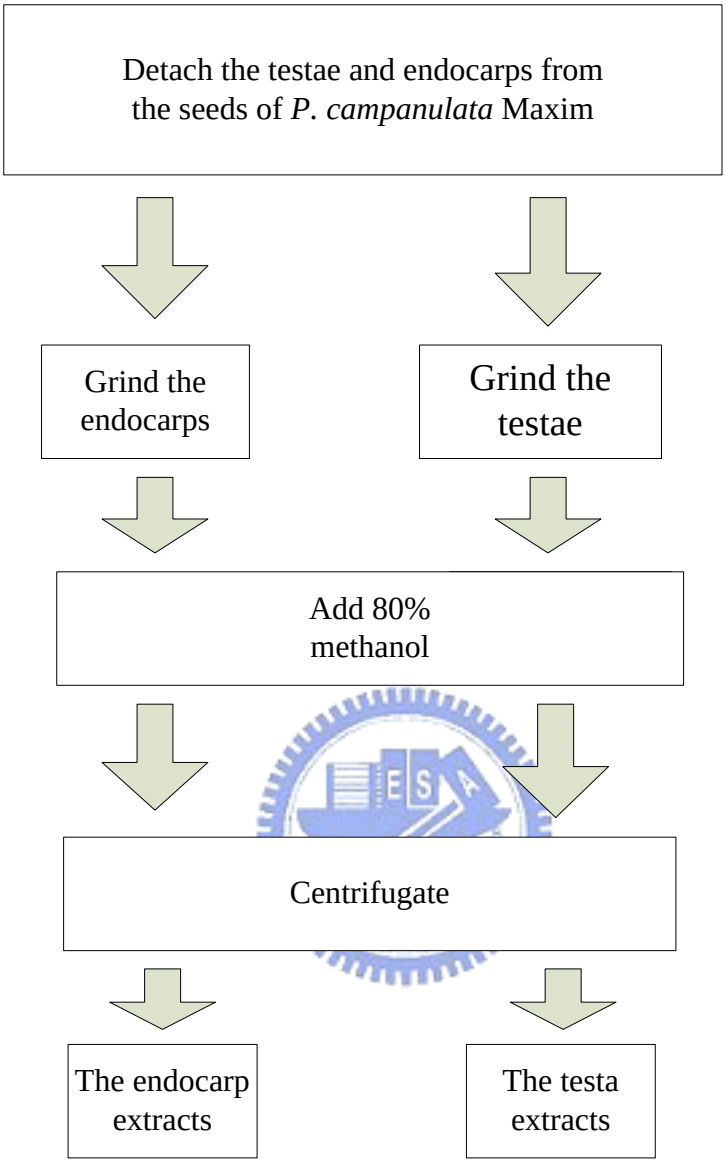
germination

	Endocarp	Testa
Water	90.0 ± 4.0	90.0 ± 4.0
Methanol	80.7 ± 3.0	80.7 ± 3.0
Fresh	0.7 ± 1.2	61.3 ± 17.9
Warm 6 weeks	42.7 ± 14.0	56.0 ± 8.7
Cold 8 weeks	10.0 ± 2.0	56.6 ± 2.3
Warm 6 and cold 8 weeks	46.0 ± 17.3	59.3 ± 15.1

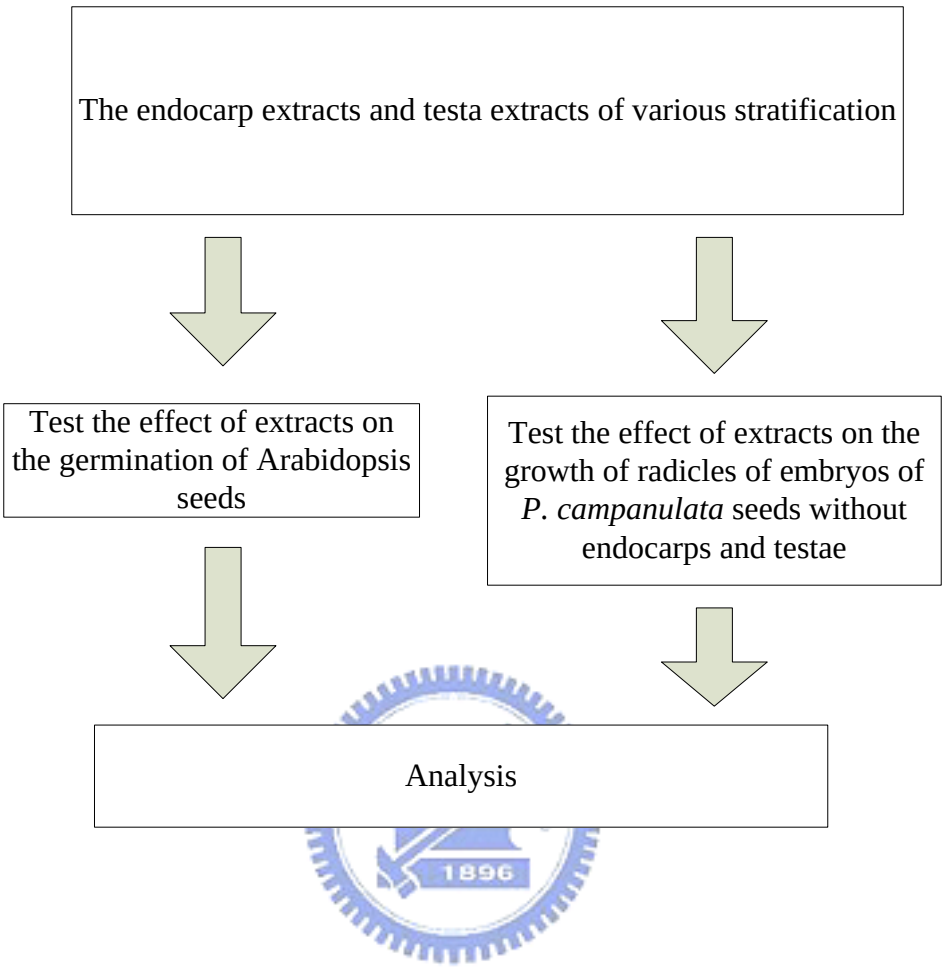
Table 4. The effect of extracts from fresh *P. campanulata* seeds on germination of *P. campanulata* embryo which was taken off endocarp and testa with cotyledons cut down to a half.

Treatment	Methanol	Testa	Endocarp
Radicle length (mm)	8.85 ± 2.32	5.48 ± 1.19	4.81 ± 0.44

Flow Chat 1

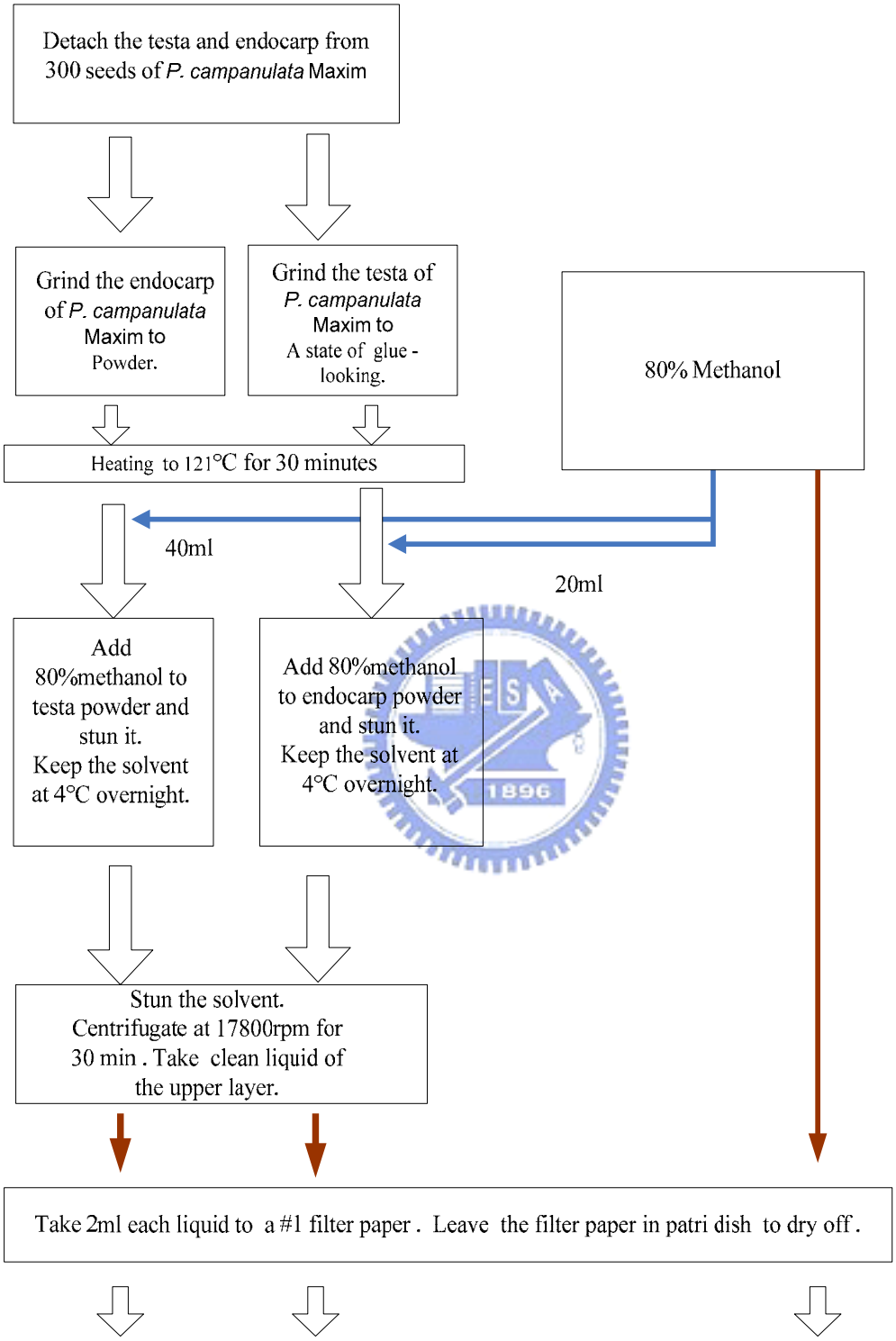

Detach the testae and endocarps from the seeds of *P. campanulata* Maxim
Grind the endocarps
Grind the testae
Add 80% methanol
Centrifugate
The endocarp extracts
The testa extracts

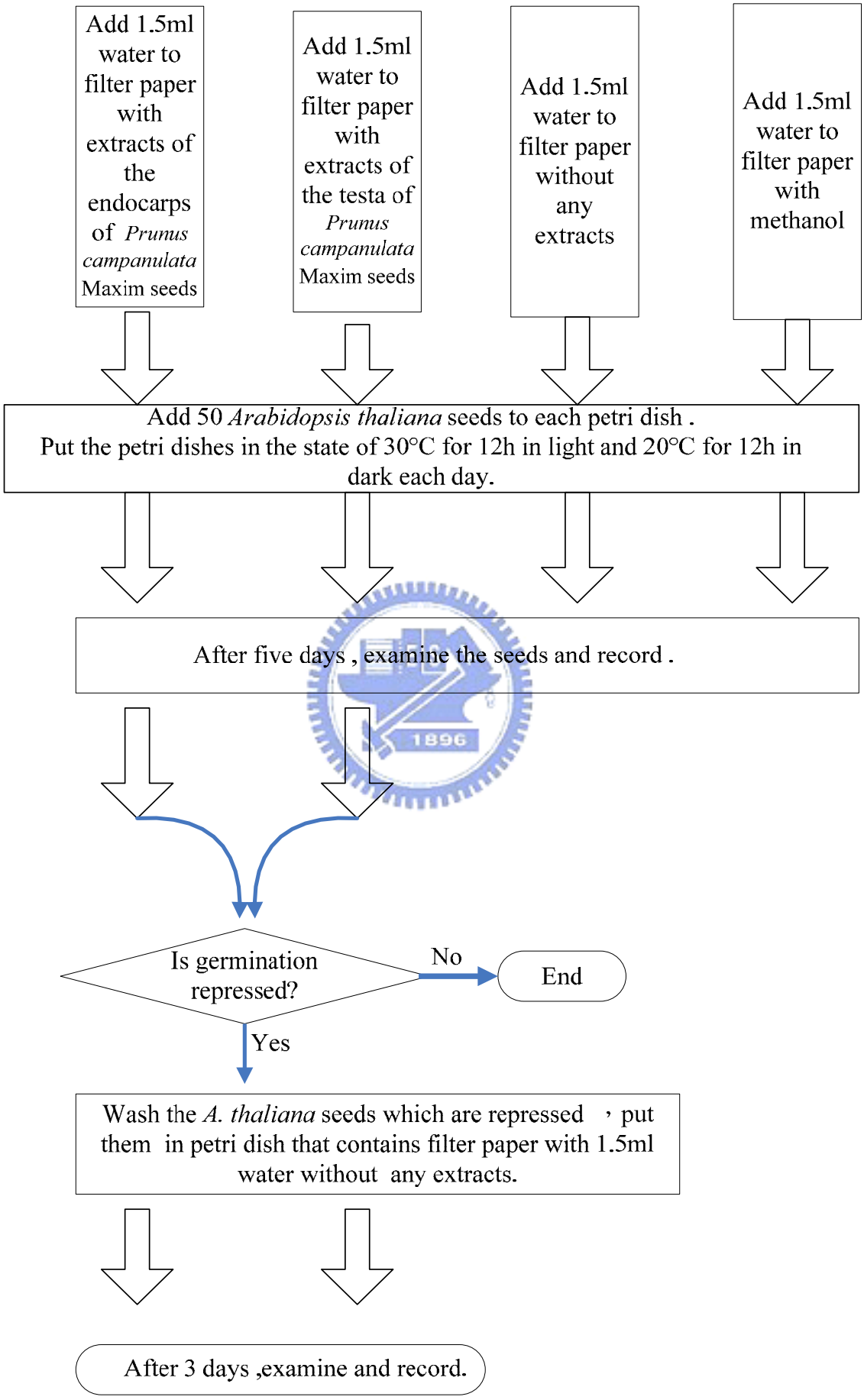
Flow Chat 2


The endocarp extracts and testa extracts of various stratification
Test the effect of extracts on the germination of Arabidopsis seeds
Test the effect of extracts on the growth of radicles of embryos of *P. campanulata* seeds without endocarps and testae
Analysis

Flow Chat 3

For explore the chemicals in the endocarps and testae of *P. campanulata* Maxim




Add 1.5ml water to filter paper with extracts of the endocarps of *Prunus campanulata* Maxim seeds
Add 1.5ml water to filter paper with extracts of the testa of *Prunus campanulata* Maxim seeds
Add 1.5ml water to filter paper without any extracts
Add 1.5ml water to filter paper with methanol
Add 50 *Arabidopsis thaliana* seeds to each petri dish .
Put the petri dishes in the state of 30°C for 12h in light and 20°C for 12h in dark each day.
After five days , examine the seeds and record .
Is germination repressed?
No
End
Yes
Wash the *A. thaliana* seeds which are repressed ，put them in petri dish that contains filter paper with 1.5ml water without any extracts.
After 3 days ,examine and record.

References

Chen SY, Chien CT, Chung JD, Yang YS, Kuo SR, (2007) Dormancy-break and germination in seeds of *Prunus campanulata* (Rosaceae) : role of covering layers and changes in concentration of abscisic acid and gibberellins. Seed Science Research 17: 21-32

Chien CT, Chen SY, Yang JC, (2002) Effect of Stratification and Drying on the Germination and Storage of *Prunus Campanulata* Seeds. Taiwan J for Sci 17(4): 413-420

Debeaujon I, Koornneef M, (2000) Gibberellin Requirement for Arabidopsis Seed Germination Is Determined Both by Testa Characteristics and Embryonic Abscisic Acid. Plant Physiology 122: 415-424

Fenner M, Thompson K, (2005) The ecology of seeds. Cambridge University Press:97-109

Finkelstein R, Gampala S, Rock C,(2002) Abscisic Acid Signaling in Seeds and Seedlings. The Plant Cell: S15-S45

Frey A, Godin B, Bonnet M, Sotta B, Marion-Poll Annie, (2004) Maternal synthesis of abascisic acid controls seed development and yield in *Nicotiana plumbaginifolia*. Planta 218: 958-964

Gubler F, Millar A, Jacobsen J, (2005) Dormancy release, ABA and pre-harvest sprouting. Current Opinion in Plant Biology 8: 183-187

Karssen CM, Brinkhorst-van der Swan DLC, Breekland AE, Koornneef M ,(1983) Induction of dormancy during seed development by endogenous abscisic acid: studies on abscisic acid deficient genotypes of *Arabidopsis thaliana* L. Heynh. Planta 157:158-165

Kermode A, (2005) Role of Abscisic Acid in Seed Dormancy. Journal of Plant Growth Regulation 24: 319-344

Koiwai H, Nakaminami K, Seo M, Mitsuhashi W, Toyomasu T, Koshiba T, (2004) Tissue-Specific Localization of an abscisic acid biosynthetic Enzyme, AAO3, in *Arabidopsis*. Plant physiology 134:1697-1707

Kushiro T, Okamoto M, Nakabayashi K, Yamagishi K, Kitamura S, Asami T, Hirai N, Koshiba T, Kamiya Y, Nambara E, (2004) The *Arabidopsis* cytochrom P450 CYP707A encode ABA 8'-hydroxylases: key enzymes in ABA catabolism. The EMBO Journal 23: 1647-1656

Lefebvre V, North H, Frey A, Sotta B, Seo M, Okamoto M, Nambara E, Marion-Poll A, (2006) Functional analysis of Arabidopsis NCED6 and NCED9 genes indicates that ABA synthesized in the endosperm is involved in the induction of seed dormancy. The Plant Journal 45: 309-319

Müller K, Tintelnot S, Leubner-Metzger G, (2006) Endosperm-limited Brassicaceae Seed germination: Abscisic Acid Inhibits Embryo-induced Endosperm Weakening of Lepidium sativum (cress) and Endosperm Rupture of Cress and Arabidopsis thaliana. Plant Cell Physiology 47(7): 864-877

Nambara E, Marion-Poll A, (2003) ABA action and interactions in seeds. Trends in Plant Science 8(5): 213-217

Okamoto M, Kuwahara A, Seo M, Kushiro T, Asami T, Hiarai N, Kamiya Y, Koshiba T, Nambara E, (2006) CYP707A1 and CYP707A2, which encode abscisic acid 8'-hydroxylases, are indispensable for proper control of seed dormancy and germination in *Arabidopsis*. Plant Physiology 141:97-107

Penfield S, Josse E, Kannangara R, Gilday A, Halliday K, Graham I, (2005) Cold and Light Control Seed Germination through the bHLH Transcription Factor SPATULA. Current Biology 15: 1998-2006

Raz V, Bergervoet JHW, Koornneef M, (2001) Sequential steps for developmental arrest in *Arabidopsis* seeds. Development 128:243-252

Rice-Evans CA, Miller NJ, Paganga G, (1997) Antioxidant properties of phenolic compounds. Trends in plant science 2:152-159

Sauter A, Davies WJ, Hartung W, (2001) The long-distance abscisic acid signal in the droughted plant: the fate of the hormone on its way from root to shoot. Journal of Experimental Botany, 52: 1991-1997

Shu BR, Chein CT, Chen YC, (2000) Germination Promotion And Storage of *Prunus campanulata* Masim Seeds. Q. Jour.Chin. For. 33(2): 283-289 (in Chinese with English abstract)

Sonia A, Bouinot D, Wagner M, Bonnet M, Sotta B, Grappin P, Jullien M,(2004) Changes in endogenous abscisic acid levels during dormancy release and maintenance of mature seeds: studies with the Cape Verde Islands ecotype, the dormant model of *Arabidopsis thaliana.* Planta 219: 479-488

Varin L, Ibrahim R, (1992) Novel Flavonol 3-Sulfotransferase. The Journal of Biological Chemistry, 267: 1858-1863

Wang H. Qi Q. Schorr P. Cutler A. Crosby W. Fowke L.(1998) ICK1, a cyclin-dependent protein kinase inhibitor from *Arabidopsis thaliana* interacts with both Cdc2a and CycD3, and its expression is induced by abscisic acid. The Plant Journal 15: 501-510

Yamasaki H. (1997) A function of colour. Trends in plant science 2:7-8

Yamauchi Y, Ogawa M, Kuwhara A, Hanada A, Kamiya Y, Yamaguchi S, (2004) Gibberellin Biosynthesis and Response Pathways by Low Temperature during Imbibition of *Arabidopsis thaliana* Seeds. The Plant Cell, 16:367-378

APPENDIX

A. Epuipments

Equipments	Company
Mixer Mill MM 301	Retsch
Filter paper (No.1 ,110mm)	Whtman

B. Methods

Z-test

- 選用z-test的原因：sample size夠大(50*3)，且假設其生理狀態爲normal distribution，並隨機挑選出sample.
- 公式

$$p = \frac{x_1 + x_2}{n_1 + n_2}$$

$$\delta = \sqrt{p * (1-p) * (\frac{1}{n_1} + \frac{1}{n_2})}$$

$$z = \frac{p_1 - p_2}{\delta}$$

When α=0.01, z ≒2.32 , if z > 2.32, the result is statically significant.

z value of all tests

germination

water		
Methanol	1.865506	1.865506
fresh endocarp	12.68903	4.723991
w6 endocarp	7.082492	5.415276
c8 endocarp	11.31371	5.330018
w6c8 endocarp	6.66973	4.985882

Intact seedcoat

water		
Methanol	-1.82841	-1.82841
fresh endocarp	-8.33106	-4.30875
w6 endocarp	-3.22223	-5.10732
c8 endocarp	-6.34959	-4.93874
w6c8 endocarp	-4.75469	-4.75469

Testa rupture

water		
Methanol	-0.60999	-0.60999
fresh endocarp	-5.83248	-1.59144
w6 endocarp	-5.58487	-1.43613
c8 endocarp	-6.71322	-1.72024
w6c8 endocarp	-3.70162	-1.27483

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