UNIVERSITA' DEGLI STUDI DI CATANIA
Dipartimento di Scienze Biologiche, Geologiche e Ambientali

DOTTORATO DI RICERCA IN
BIOLOGIA EVOLUZIONISTICA E DELL'AMBIENTE
Coordinatore: Prof. Salvatore Saccone

GIUSEPPE DIEGO PUGLIA

Meccanismi fisiologico-molecolari di germinazione in specie spontanee

Tesi di dottorato

Tutor: Prof. Pietro Pavone
Co-Tutor: Dr. Peter Toorop

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Physiological and molecular mechanisms of seed germination in weed species

Ph.D. thesis

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Abstract

Seed dormancy provides a mechanism for plants to delay germination until conditions are optimal for survival of the next generation. Knowledge of dormancy release and dormancy induction requirements is important to increase our understanding about the entire process. Furthermore, assess the association between gene expression patterns and physiological phase in dormancy is a major goal to allow a positive definition of this condition.

In *Chrysanthemum coronarium* var. *concolor* and var. *discolor*, anatomical and germination analyses showed that non-deep physiological dormancy (PD) is the more plausible type of dormancy for both of them. The genetic composition of *Chrysanthemum coronarium* varieties was characterized using Inter Simple Sequence Repeat (ISSR) PCR together with a DNA-Barcoding approach.

Seed priming treatments were used in a non-dormant species, *Leucanthemum vulgare*, to induct secondary dormancy at specific temperature range and a long-priming treatment was used for its release. Fluridone and giberellic acid were tested to investigate the metabolic pathway involved in the onset of secondary dormancy and in its maintenance.

Five probable housekeeping genes, useful as reference genes for quantitative PCR (qPCR) analysis in seed tissue, were identified in *Chrysanthemum coronarium* and in *Leucanthemum vulgare*, isolated and confirmed with comparative analysis with homologous genes in model species. One radicle-protrusion associated gene, *EXP (Expansin)*, was identified in the two species, isolated and confirmed. Three dormancy-associated genes, *DOG1 (Delay Of Germination 1)*, *FLC (Flowering Locus C)*, *HUB2 (Histone Mono-Ubiquitination 2)* were identified in *Chrysanthemum coronarium* and in *Leucanthemum vulgare*.

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Introduction

Seed germination

Seeds are the mobile phase of the plant's life cycle, vegetative development is suspended as seeds transport the plant's genetic complement through space and time.
Seeds are mostly shed from the mother plant in a dry state in which the seed tissues (embryo, covering layers) are preserved at low water content. Seed germination commences with the uptake of water by the dry seed, followed by embryo expansion growth (Hilhorst 2010). Under favourable conditions rapid expansion growth of the embryo culminates in rupture of the covering layers and emergence of the radicle.

Water imbibition and metabolism reactivation

Uptake of water by a mature dry seed is triphasic Figure 1, with a rapid initial uptake (phase I) followed by a plateau phase (phase II). A further increase in water uptake occurs only after germination is completed, as the embryonic axes elongate. Because dormant seeds do not complete germination, they cannot enter phase III. The influx of water into the cells of dry seeds during phase I results in temporary structural perturbations, particularly to membranes, which lead to an immediate and rapid leakage of solutes and low molecular weight metabolites into the surrounding imbibition solution (J. D. Bewley 1997) (Crowe 1992).
Upon imbibition, the quiescent dry seed rapidly resumes metabolic activity. The structures and enzymes necessary for this initial resumption of metabolic activity are generally assumed to be present within the dry seed, having survived, at least partially intact. Reintroduction of water during imbibition is sufficient for metabolic activities to resume, with turnover or replacement of components occurring over several hours as full metabolic status is achieved Figure 1. One of the first changes upon imbibition is the resumption of respiratory activity, which can be detected within minutes. After a step initial increase in oxygen consumption, the rate declines until the radicle penetrates the structures. At this time, another burst of respiratory activity occurs: the glycolytic and oxidative pentose phosphate pathways both resume during phase I, and the Kreb's cycle enzymes become activated (Botha 1992) (J. D. Bewley 1994).

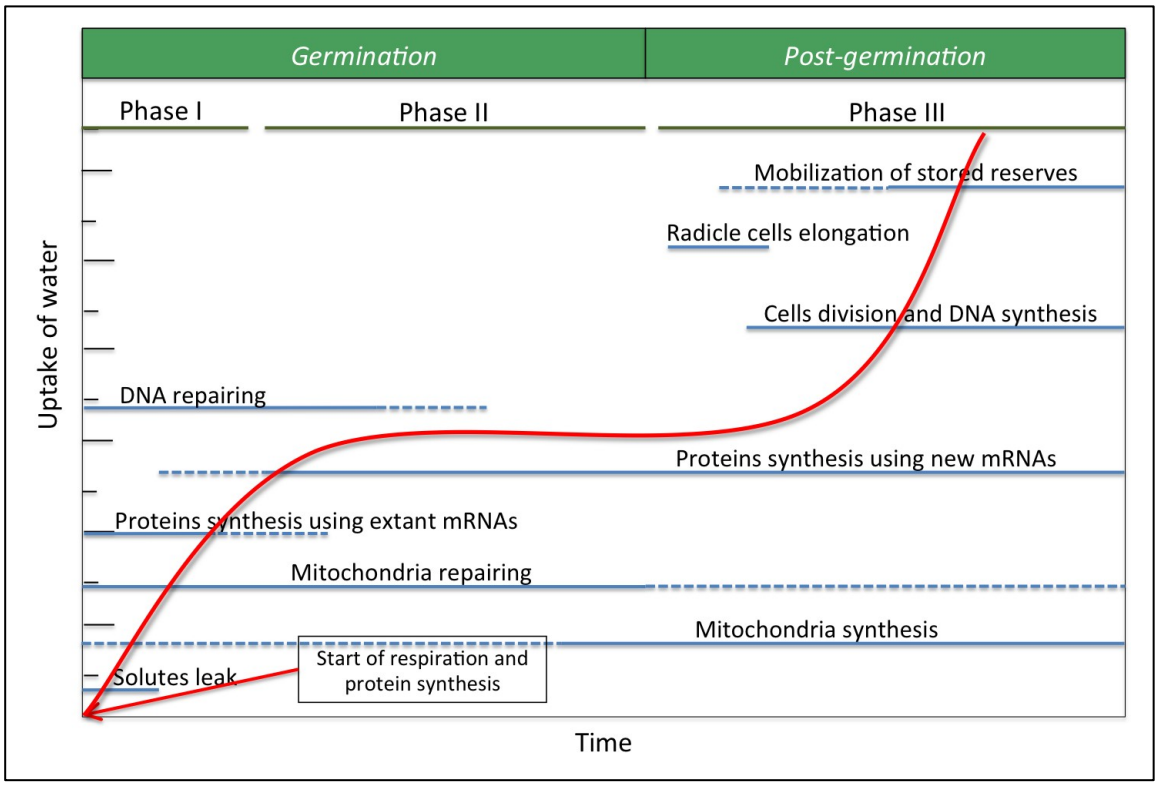

Figure 1 Time course of major events associated with germination and subsequent post-germination growth

Protein synthesis

All of the components necessary for the resumption of protein synthesis upon imbibition are present within the cells of mature dry embryos, although polysomes are absent. However, within minutes of rehydration there is a decline in the number of single ribosomes as they become recruited into polysomal protein-synthesizing complexes. Initial protein synthesis is dependent on extant ribosomes, but newly synthesized ribosomes are produced and used within hours of initial polysome assembly (Dommes 1990). Preformed mRNAs are also present within the dry embryo.

Some of these are residual messages associated with previous developmental processes (Comai 1990) (Lane 1991) and may be used transiently during early germination Figure 1. Messages encoding proteins that are important during seed maturation and drying, such as *late embryogenesis abundant* (LEA) proteins, are likely to be degraded rapidly upon imbibition (Jiang 1994) (Han 1996). Conversely, those encoding proteins required during early germination (e.g., ribosomal protein messages) are replaced by identical messages at later times, with protein synthesis becoming more dependent on the new transcripts with time (Beltran-Petia 1995) (see Figure 1).

New mRNAs are transcribed as germination proceeds. The majority of these are likely to encode proteins essential for the support of normal cellular metabolism, that is, "growth maintenance" reactions that are not restricted to germination (J. D. Bewley 1990). Nevertheless, some changes in embryo mRNA populations and synthesized proteins do occur during germination of several species of monocots (e.g., maize; (Sánchez-Martinez 1986), dicots, e.g. peas (Lalonde 1986), and conifers, e.g. loblolly pine (Mullen 1996)).

Radicle extension

With few exceptions, radicle extension through the structures surrounding the embryo is the event that terminates germination and marks the commencement of seedling growth. This extension may or may not be accompanied by cell division. Two discrete phases of DNA synthesis occur in the radicle cells after imbibition Figure 1. The first takes place soon after imbibition and probably involves the repair of DNA damaged during maturation drying and rehydration as well as the synthesis of mitochondrial DNA. DNA synthesis associated with post-germinative cell division accounts for the second phase Figure 1 (Zlatanova 1987) (Osborne 1994).

Extension of the radicle is a turgor-driven process that requires yielding of walls in those cells of the embryonic root axis that lie between the root cap and the base of the hypocotyl. There are three possible reasons for the commencement of radicle growth. One possibility is that late during germination, the osmotic potential (ψ_π) of the radicle cells becomes more negative because of the accumulation of solutes, perhaps as a result of the hydrolysis of polymeric reserves present within the radicle cells themselves. The decrease in ψ_π would lead to increased water uptake, and the resulting increase in turgor would drive cell extension. A second possibility is that extensibility of the radicle cell walls allows for their elongation. Whether the mechanisms by which cells of the radicle become more extensible differs from those in other tissues is not known. Cell wall loosening may result from the cleavage and re-joining of xyloglucan molecules that tether adjacent cellulose microfibrils, which would permit expansion by microfibril separation. The activity of xyloglucan endotransglycosylase (XET), an enzyme capable of reversibly cleaving xyloglucan molecules, increases in the apical region of maize seedling roots during their elongation (Wu 1994), but this increase occurs after germination is completed. Alternative candidates for cell wall-loosening proteins are the *expansins*, which have the ability to disrupt the hydrogen bonds between cell wall polymers (e.g. matrix polysaccharides and cellulose microfibrils). Expansins have been strongly implicated in the expansion of

cucumber hypocotyls (McQueen-Mason 1995) (Cosgrove 1997) and in the coleoptile elongation in rice cultivars (Magneschi 2009).
However, neither of these cell wall-loosening proteins has been reported in germinating seeds. Moreover, both XET and expansin activities in seedlings appear to be enhanced by auxin, which is generally regarded as ineffective in promoting seed germination, and XET activity in maize seedling roots is also enhanced by abscisic acid (ABA), a potent inhibitor of embryo radicle elongation! A third possibility is that the seed tissues surrounding the radicle tip weaken, thus allowing the tip to elongate. Because there are no changes in cell ψ_π before radicle growth commences, it is axiomatic that the turgor potential (ψ_ρ) of the radicle cells is sufficient to drive their elongation if there is little or no restraint exerted by the surrounding structures.

In many germinating seeds, including those of rape, the testa splits during imbibition, and it is only the rigidity of the radicle cell walls that restrains growth (Schopfer 1985) (Figure 2). As the walls yield during the initial stages of radicle elongation, there is a decline in cell ψ_ρ. Conversely, in other seeds, ψ_ρ alone is insufficient to drive wall extension, and there is a severe constraint on radicle cell growth imposed by the surrounding structures. In lettuce, tobacco, and tomato seeds, the endosperm is the constraining structure, whereas in muskmelon it is the perisperm. A reduction in the resistance of these enclosing structures is necessary for germination to be completed. Measurements have revealed a decline in the mechanical resistance of the structures covering the embryo root cap at the time of radicle emergence in the endosperm of pepper seeds (Watkins 1983) and in endosperm cap in tomato (Toorop 2000). This decline in resistance is likely to be achieved by cell wall hydrolases, such as hemicellulases, produced within and secreted by the endosperm itself.

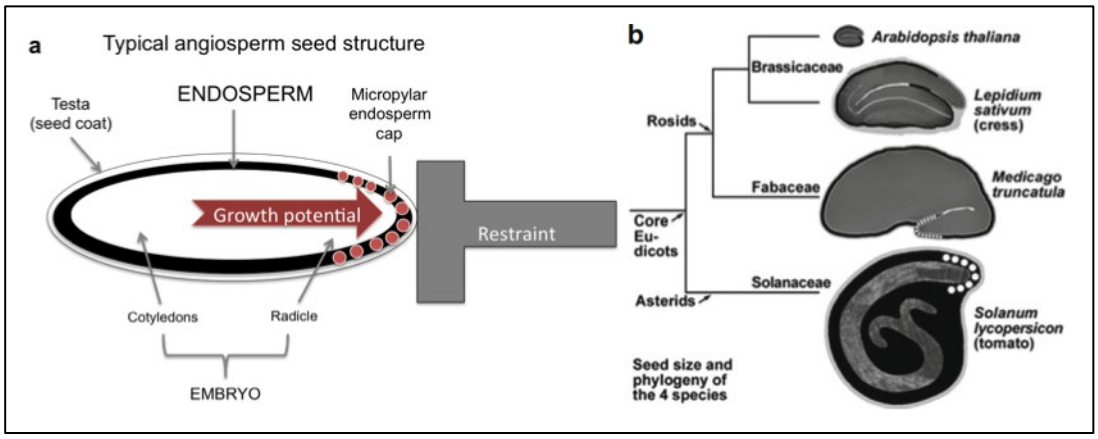

Figure 2 Seed structures, sizes and phylogenetic relationships of model and crop species. (a) Generalised structure of an angiosperm (eudicot) seed with EMBRYO and ENDOSPERM as the two important seed components. The direction of embryo growth (arrow 'Growth potential') that results in germination (rupture of the endosperm) and repressive function of endosperm (block 'restrain') are shown. (b) Phylogenetic relationship and seed size comparison for *Arabidopsis*, *Lepidium*, *Medicago*, and tomato.

Seed dormancy

Germination timing is a plant trait with the highest selection pressure by the environment and has, during seed evolution, led to a connected second key trait: seed dormancy. This can be defined as the (temporary) incapacity of a viable imbibed seed to germinate under favourable conditions.

Virtually all of the cellular and metabolic events that are known to occur before the completion of germination of non-dormant seeds also occur in imbibed dormant seeds; indeed, the metabolic activities of the latter are frequently only subtly different from those of the former. Hence, a dormant seed may achieve virtually all of the metabolic steps required to complete germination, yet for some unknown reason, the embryonic axis (i.e., the radicle) fails to elongate (J. D. Bewley 1997).

Seed dormancy could be considered simply as a block to the completion of germination of an intact viable seed under favourable conditions. In the review 'Seed dormancy and the control of germination' (W. E.-M. Finch-Savage 2006) they present an integrated view across the evolution, molecular genetics, physiology, biochemistry, modelling and eco-physiology of the control of seed germination by dormancy in an attempt to draw together these linked, but often separate disciplines.

A more sophisticated and experimentally useful definition of dormancy has been proposed by Baskin J.M. (J. M. Baskin 2004): a dormant seed does not have the capacity to germinate in a specified period of time under any combination of normal physical environmental factors that are otherwise favourable for its germination, i.e. after the seed

becomes non-dormant. On the other hand, a completely non-dormant seed has the capacity to germinate over the widest range of normal physical environmental factors possible for the genotype. For the species survival, the role of seed dormancy is to spread germination across time, but in synchrony with the seasons to avoid unfavourable conditions for plant establishment (C. C. Baskin 1998) (Rodríguez 2011).

Seed physiology literature discriminates two types of dormancy focussing on the timing of the onset respect to the developmental stage of seed: *primary dormancy* (PD) refers to the type of dormancy that occurs prior to dispersal as part of the seed developmental program, whereas *secondary dormancy* (SD) refers to the acquisition of dormancy in a mature seed after imbibition as a result of the lack of proper conditions for germination (Amen 1968) (Hilhorst 2010).
Nikolaeva (Nikolaeva 1969) devised a dormancy classification system reflecting that dormancy is determined by both morphological and physiological properties of the seed. Based on this scheme, C. Baskin and J. Baskin (J. M. Baskin 1998) have proposed a comprehensive classification system which includes five classes of seed dormancy. The system is hierarchical with these five classes further divided into levels and types Baskin and Baskin (J. M. Baskin 2004).

Physiological dormancy (PD)

PD (dormancy class A according to Baskin and Baskin, 2004) is the most abundant form and is found in seeds of gymnosperms and all major angiosperm clades. It is the most prevalent dormancy form in temperate seed banks and the most abundant dormancy class "in the field". PD is also the major form of dormancy in most seed model species "in the lab", including *Arabidopsis thaliana, Helianthus annuus, Lactuca sativa, Lycopersicon esculentum, Nicotiana spp., Avena fatua*, and several cereals.

PD deep

Embryos excised from PD-deep seeds either do not grow or will produce abnormal seedlings. GA treatment does not break their dormancy. Ca. 3-4 months of cold (subtype a) or warm (subtype b) stratification are required before germination can take place. Examples: *Acer platanoides* (PD deep subtype a) Aceraceae (W. E. Finch-Savage 1998); *Leptecophylla tameiameiae* (PD deep subtype b) (C. C. Baskin 2005) (Blumenthal 1986).

PD intermediate

Embryos excised from PD-intermediate seeds produce normal seedlings. GA promotes germination in some (but not all) species. Seeds require 2-3 months of cold stratification. Dry storage (after-ripening) can shorten the cold stratification period. Example: *Acer pseudoplatanus* (PD intermediate), Aceraceae (W. E. Finch-Savage 1998).

PD non-deep

The great majority of seeds have non-deep PD. Embryos excised from these seeds produce normal seedlings. GA treatment can break this dormancy and depending on species dormancy can also be broken by scarification, after-ripening in dry storage, and cold (0-10° C) or warm (>15° C) stratification. Based on patterns of change in physiological responses to temperature five types of non-deep PD can be distinguished (see Figure 3). Most seeds belong to type 1 or 2, in which the temperature range at which seed germination can occur increases gradually during the progression of non-deep dormancy release from low to higher (type 1, e.g. *Arabidopsis thaliana*) or from high to lower temperature (type 2, e.g. *Helianthus annuus*). In addition, the sensitivity of the seeds to light and GA increases as non-deep PD is progressively released. The molecular mechanisms of Arabidopsis seed dormancy cycling have been investigated by transcriptome analyses (Cadman 2006).

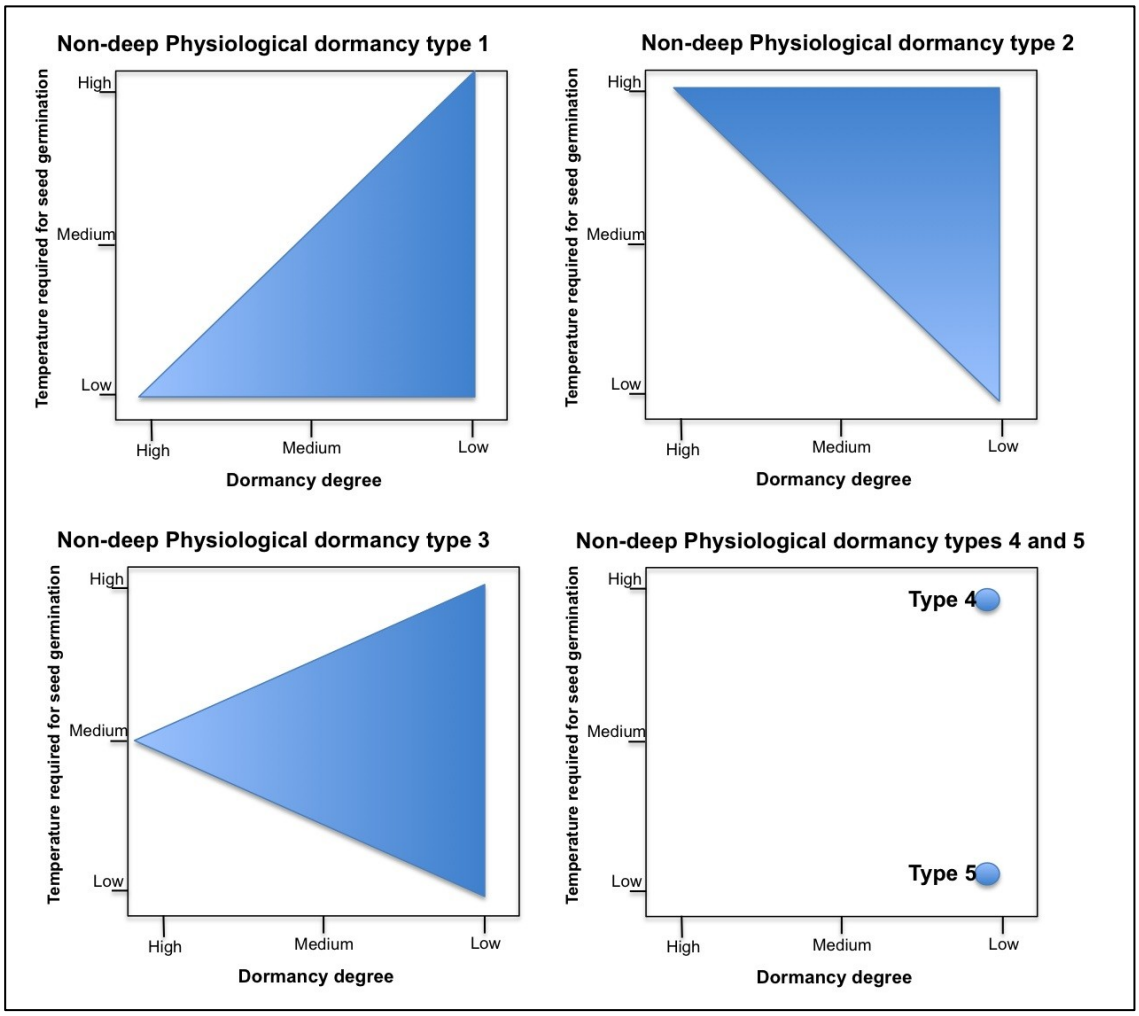

Figure 3 Five types of non-deep physiological seed dormancy (PD) according to Baskin and Baskin (2004). Type 1: Temperature range for germination increases during dormancy release as a continuum from low to high, example: *Arabidopsis thaliana*; Type 2: Temperature range for germination increases during dormancy release as a continuum from high to low, example: *Helianthus annuus*; Type 3: temperature range for germination increases during dormancy release as a continuum from medium to high and low, example: *Aster ptarmacoides*. Type 4 and 5: No temperature and dormancy continuum, non-dormant seeds germinate only at high temperature (Type 4), example: *Callicarpa americana* or low temperature (Type 5), example: *Gentianella quinquefolia.*

Physical restriction and embryo growth potential

Within the non-deep physiological dormancy the removal or disruption of structures that covers the embryo, such as endosperm, seed coat, fruits coats, could results in germination. Embryo or seed covers could, indeed, exert an inhibitory effect of mechanically restricting embryo or seed growth.

The force required to break seed coats ranges from 9.9MPa in *Pancratium marithimum* to 133.2MPa in *Iris lorteti* (Blumenthal 1986). Thus germination could be prevented because embryos lack a sufficient growth potential to break open seed coat or other structures (Khan 1982). Depending on the species, cold stratification (Carpita 1983), giberellic acid (J. M. Baskin 1971), incubation temperatures (Juntilla 1973), light (Scheibe 1965), or

darkness (S. Chen 1968) may increase the growth potential of the embryo enough for the radicle to push through the seed coat and thus for seed to germinate.
Scarification of positively photoblastic seeds of cucumber and sunflower resulted in about 50% increase of germination in light at 22° C in 0.45*M* (1.13MPa) and 0.3*M* (0.75MPa) mannitol solutions, respectively, indicating that removal of the mechanical constraint allowed seeds to germinate at an increased osmotic potential (McDonough 1967). In tomato the endosperm cap weakening is a biphasic process correlated with endo-β-mannase activity for the first phase and controlled by ABA in the latter one (Toorop 2000).

Morphological dormancy (MD)

MD (dormancy class B according to Baskin and Baskin, 2004) is evident in seeds with embryos that are underdeveloped (in terms of size), but differentiated (e.g. into cotyledons and hypocotyl-radical). These embryos are not (physiologically) dormant, but simply need time to grow and germinate. This group does not include seeds with undifferentiated embryos. Example: *Apium graveolens* (Apiaceae).

Morphophysiological dormancy (MPD)

MPD (dormancy class C according to Baskin and Baskin, 2004) is also evident in seeds with underdeveloped (in terms of size) embryos, but in addition they have a physiological component to their dormancy. These seeds therefore require a dormancy-breaking treatment, e.g. a defined combination of warm and/or cold stratification which in some cases can be replaced by GA application. In MPD-seeds embryo growth/emergence requires a considerably longer period of time than in MD-seeds. Seeds with undifferentiated embryos like the Orchidaceae also have a morphological and a physiological component of dormancy, but they are not considered in this classification scheme (J. M. Baskin 2004).

Physical dormancy (PY)

PY (dormancy class D according to Baskin and Baskin, 2004) is caused by one or more water-impermeable layers of palisade cells in the seed or fruit coat (J. M. Baskin 2000), (C. C. Baskin 2003), (J. M. Baskin 2004). This palisade layer(s) prevent water uptake by their physical arrangement as a tissue and the chemical coatings/impregnates of the cells (heavy lignification, suberin-cutin matrix, waxes) Figure 4. In seeds PY-seeds, prevention of water uptake develops during maturation drying and the covering layer(s) control water

movement (often associated with hard seed coat). Seeds will remain dormant until some factor(s) render the covering layer(s) permeable to water. In nature, these factors include high temperatures, widely fluctuating temperatures, fire, drying, freezing/thawing and passage through the digestive tracts of animals. In seed technology, mechanical or chemical scarification can break PY dormancy. Once PY is broken, i.e. the seed or fruit coat becomes permeable to water, the seeds can germinate over a wide range of ambient conditions. Unlike PD-seeds, which may re-enter (secondary) dormancy after primary dormancy is broken, once the coat of PY-seeds becomes permeable it generally cannot revert to complete impermeability. Thus, the timing of dormancy break in nature is a more critical event in the life cycle of plants with PY, than it is in those with PD. The mechanism for PY-break must therefore be fine-tuned to the environment.

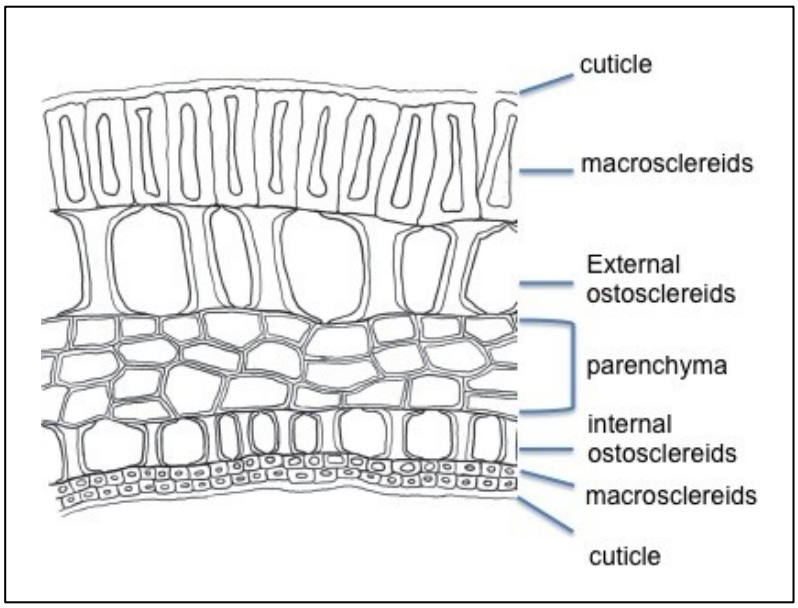

Figure 4 Testa (seed coat) structure of a typical legume seed (Fabaceae). Water impermeability is due to a palisade layer of macrosclereids (Malphighian cells) in the exotesta.

Combinational dormancy (PY+PD)

Combinational dormancy, PY+PD (dormancy class E according to Baskin and Baskin 2004), is evident in seeds with water-impermeable coats (PY) combined with physiological embryo dormancy (PD non-deep). Within the Ancardiaceae the genus *Rhus* includes species with PY+PD- dormancy (e.g. *Rhus aromatica*) and species with only PY-dormancy (e.g. *Rhus glabra*). Embryos of freshly harvested mature PY+PD-dormant seeds of winter annuals, e.g. *Geranium* spp. (Geraniaceae, Rosids) and *Trifolium* spp. (Fabaceae, Rosids), have some conditional dormancy (PD non-deep). This PD is released during after-ripening storage, even of the seed coat remains water impermeable (PY). Embryos of PY+PD-

dormant *Ceanothus* spp. (Rhamnaceae, Rosids) are more deeply dormant (but still PD non-deep). In this case PY is released first and the subsequent water uptake during imbibition is followed by the release of the embryo dormancy (PD non-deep) during a few weeks of cold stratification. Thus, release from PY and PD of PY+PD-dormant seeds seems to be by independent events and the timely order can be species-specific.

Molecular aspects of seed dormancy

Despite the obvious importance of dormancy cycling in the natural environment (J. M. Baskin 1998), very little is known about its regulation at a molecular level. In contrast, a great deal is known about mechanisms that influence dormancy loss in short-term laboratory experiments (W. E.-M. Finch-Savage 2006), (Finkelstein 2008), (Holdsworth 2008) that inform us about dormancy in the context of crop seeds.

Recent -omics studies of the transcriptome (Cadman 2006) (W. E. Finch-Savage 2007) and proteome (Gallardo 2002) (Rajjou 2004) show that seeds change and remain responsive at a molecular level in both the imbibed and the dry state. A dynamic balance of the hormones abscisic acid (ABA) and gibberellic acid (GA), resulting from both synthesis and catabolism, is thought to be central to dormancy and the control of germination completion (radical emergence through the seed coat) (W. E.-M. Finch-Savage 2006). Regulation of dormancy status results from the response to this balance through hormone-signaling networks that influence sensitivity to ABA and GA. The influence of other hormones, such as ethylene (Linkies 2009), can be significant, but in general their influence operates through the ABA/GA balance.

Recently, the seed transcriptome, and in particular those genes involved in the ABA/GA balance, were shown to be sensitive to maturation temperature, which is important in setting dormancy status before shedding from the mother plant (Kendall 2011). Genetic approaches have led to the identification of specific loci correlated with altered dormancy, and -omics approaches in many species have identified numerous transcripts and proteins correlated with dormant versus non-dormant seeds. Studies on model species, in fact, leaded to the identification of the *DELAY OF GERMINATION 1* (*DOG1*) gene involved in the control of seed dormancy (L. J. Bentsink 2006) or the *HISTONE MONOUBIQUITINATION* (*HUB1*) locus and a close homolog, *HUB2*, which mutants resulted in decreased dormancy (Liu 2007).

Dormancy release

In most angiosperm species, primary dormancy is gradually lost after the seed is ready for dispersal (in wild species) or harvest during a post-maturation or after-ripening period. This process takes place in seeds during dry storage or in buried seeds in the soil as well, although these different environmental conditions can have great impact on the rate at which dormancy is terminated. Release of primary dormancy occurs at different rates depending mainly on the genotype (Probert 2000), but time is needed in many species for their seeds to be able to germinate (although some hard-coated seeds depend absolutely on chemical or mechanical scarification). Even in species that depend on the presence of light for germination (like many Solanaceae), sensitivity to light also varies with time after seed dispersal, and seed germination eventually becomes light independent (Probert 2000). So, the time lapse required by a seed population to lose dormancy and be able to undergo rapid germination immediately after sowing is a character that varies greatly not only between species, but also between different genotypes (i.e., cultivars, inbred lines, etc.) of the same species (Rodríguez 2011).

Dry after-ripening

During dry storage, seeds undergo physiological changes, which are often reflected in a decline in the level of innate dormancy. Accompanying these dry after-ripening changes, germination requirements usually become less specific (Probert 2000).

After-ripening, i.e. a period of usually several months of dry storage at room temperature of freshly harvested, mature seeds, is a common method used to release dormancy and to promote germination (J. D. Bewley 1997) (W. E.-M. Finch-Savage 2006) (Kucera 2005) (Leubner-Metzger 2003). Seed after-ripening can be characterized by (Leubner-Metzger 2005):

- A widening of the temperature range for germination.
- A decrease in ABA level and sensitivity and an increase in GA sensitivity or loss of GA requirement.
- A loss of light-requirement for germination in seeds that do not germinate in darkness.
- An increase in seed sensitivity to light in seeds that do not germinate even with light.
- A loss of the requirement for nitrate.

- An increase of germination velocity. During tobacco seed after-ripening this is evident by a promotion of testa rupture and endosperm rupture.

The parameters that determine seed after-ripening are moisture and oil contents, seed covering structures, and temperature (Manz 2005).
However, for species adapted to regions of seasonal drought and dry soils – for example, winter annuals – physiological changes recorded during dry storage reflect a natural mechanism, which governs the timing of germination in the wild. When seeds of winter annuals are in a state of conditional dormancy, shortly after dispersal, germination is characteristically restricted to a narrow range of low temperatures (P. A. Thompson 1970) (J. M. Baskin 1971) (J. M. Baskin 1978) (Pemadasa 1975).

Warming

In contrast to the immense literature on low-temperature effects, there have been far fewer reports of the effect of warm temperatures on changes in germination behaviour. However, it is clear that, like chilling, warm temperature pre-incubation of imbibed seeds can both release and induce dormancy (Probert 2000). In summer annuals in general, low winter temperatures release dormancy, whereas high summer temperatures induce dormancy (J. M. Baskin 1977) (J. M. Baskin 1987), while in winter annuals the reverse is true (J. M. Baskin 1978) (J. M. Baskin 1978). In a study on germination in desert perennial shrub species from South Africa, Gutterman (Gutterman 1990) demonstrated that the effect of pre-incubating seeds at 45° C for 24 h depended on whether plants originated from areas receiving winter or summer rain. In the former, germination was adversely affected, whereas, in the latter, germination was stimulated by high-temperature treatment.

Seed priming

Seed priming is the induction of a particular physiological state in plants by the treatment of natural and synthetic compounds to the seeds before germination (Jisha 2013). For priming the seeds, seeds are partially hydrated until the germination process begins, but radical emergence does not occur (Bradford 1986).

Seed priming is widely used to synchronize the germination of individual seeds (Taylor 1990). Improved seed invigoration techniques are known to reduce emergence time, accomplish uniform emergence, and give better crop stand in many horticultural and field crops (Ashraf 2005). Seed-priming technology has twofold benefits: enhanced, rapid and uniform emergence, with high vigor and better yields in vegetables and floriculture (Bruggink 1999) and some field crops (S. M. Basra 2005) (Kaur, Seed priming increases crop yield possibly by modulating enzymes of sucrose metabolism in chickpea. 2005). According to McDonald *et. al.* (McDonald 2000), primed seeds acquire the potential to rapidly imbibe and revive the seed metabolism thus enhancing the germination rate.

Primed seeds usually exhibit an increased germination rate, greater germination uniformity, and at times, greater total germination percentage (S. M. Basra 2005). The various approaches include hydropriming, osmopriming, chemical priming, hormonal priming, biological priming, redox priming, solid matrix priming, etc.

Hydropriming

Hydropriming has been reported to be a simple, economical and a safe technique for increasing the capacity of seeds towards osmotic adjustment, enhancing seedling establishment and crop production under stressed conditions (Kaur 2002). In this priming method, the seeds are immersed in sterilized distilled water kept at appropriate temperature and the duration of hydropriming is determined by controlling seed imbibition during germination (Kaya 2006). It is absolutely necessary to dry the seeds after soaking, as storing of improperly dried seeds will do more harm than good (Thomas 2000). After soaking, seeds were re-dried to their original weight with forced air under shade (Bennett 1987). In hydropriming, the advantageous fact is the enhancement of physiological and biochemical events taking place in seeds even when the germination is suspended by low osmotic potential and negligible matric potential of the imbibing medium (S. M. Basra 2003). Moreover, the protoplasm of hydroprimed seeds/plants is found to have a lower viscosity and exhibit higher permeability to water and nutrients and also hold water against

dehydrating forces (Thomas 2000). Increase in the seedling growth correlated with higher water uptake by primed seeds is the predominant feature in the case of hydropriming (Yagmur M. 2008). Hydropriming was found to be the most effective method for improving seed germination of onion, especially when the seeds were hydrated for 96 h compared to 48 h (Caseiro 2004). Priming decreased the temperature optimum and ceiling temperature for germination and also helped in advancing the germination time and did not decrease the final percentage emergence (W. E. Finch-Savage 2004).

Osmopriming

Osmoconditioning or osmopriming is the soaking of seeds in aerated, low-water-potential solutions. Osmopriming essentially exposes seeds to low external water potential to restrict the rate and extent of imbibition. The process of osmopriming is akin to a prolonged early imbibition of seeds that sets in motion a gradual progression of various pre-germinative metabolic activities. Thus, it is helpful to use osmopriming as a model to study the transition of seeds from a dry and physiologically quiescent to a hydrated and physiologically active state (K. A. Chen 2011).

A variety of chemicals are used to create low-waterpotential solutions. Polyethylene glycol (PEG) is more commonly used as water potential lowering agent because of its nontoxic nature and large molecular size, which lowers water potential without penetrating into the seeds during soaking (Thomas 2000). Jett *et al.* (Jett 1996) explained that, osmopriming in comparison with hydropriming can preserve plasma membrane structure and cause seeds to have better responses to germination traits because of controlled long hydration in seeds. In rice, the physiological changes produced by osmohardening enhanced the starch hydrolysis and made more sugars available for embryo growth, vigorous seedling production and later on improved allometric, kernel yield and quality attributes (Farooq 2006). Rouhi *et al.* (Rouhi 2011) suggested that different priming techniques (hydro and osmopriming) had a varying effects on germination on each of the four grass species (*Bromus inermis, Festuca arundinacea, Agropyron elongatum* and *Festuca ovina*) and the result showed that, for most evaluated germination parameters, osmopriming treatment (with PEG) was more useful technique to reduce abiotic stress than hydropriming treatment.

It is known that seed priming can activate these signaling pathways in the early stages of growth and result in faster plant defense responses. The exact molecular mechanism

behind priming is not completely known, it is speculated that sensitization was associated with accumulation of inactive signaling proteins in primed cells.

According to Nascimento and West (Nascimento 1998), the increase in germination percentage/seed vigour of primed seeds is due to reserve mobilization of food materials, activation and re-synthesis of some enzymes and also due to the increased DNA and RNA synthesis. Priming is also capable of repairing some of the damages due to seed erosion, which in turn results in increased vigour of primed seeds (Arif 2008). Seed priming affects the lag phase of seed germination and thus causes early DNA replication (Bray 1989).

In contrast to the immense literature on positive effects of priming, to date there are no reports recording a negatively interference of this treatment with germination in non-dormant seeds. In Amritphale *et.al.* (D. S. Amritphale 2000) a mechanism to induce dormancy is described which take into consideration a seed incubation in an osmoticum and the usage of far red light for 15 min; in this report the presumed induction system regarded the change in fluidity of cell membranes which are affected by a non-complete hydration.

Chrysanthemum coronarium

Species description and distribution

Chrysanthemum coronarium L. (family Asteraceae; tribe Anthemideae; genus *Chrysanthemum*) is an aromatic heterocarpic winter annual 20-60 cm high with a straight, hairless and branched trunk; leaves are light green, and deeply lobate. The chorological type is Steno-Mediterranean, see Figure 5 (Pignatti 1982) (Meusel 1965).

It is usually found as weed in diverse crops, along roadsides, on field margins and on abandoned fields Figure 6. Although essentially Mediterranean this species is nowadays naturalized far beyond their 'native' range and since its abundance and persistence it was reported as invasive plant species (Cook 2011) (Schaefer 2011). In Sicily, his phytosociological association was described by earlier authors (Ferro 1980) (Brullo 1985) (Brullo 2001) (Ferro 2004).

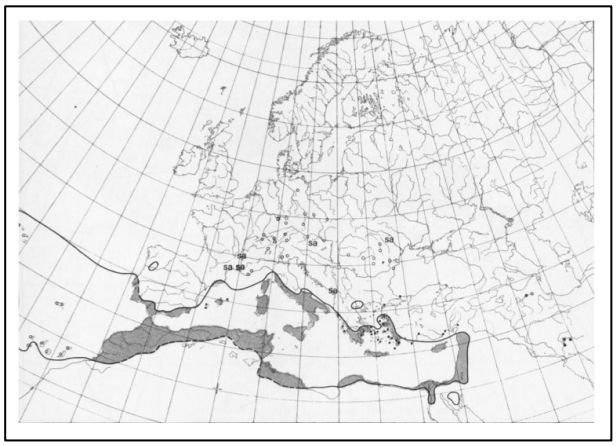

Figure 5 *Chysanthemum coronarium* distribution according to (Meusel 1965).

Dumont d'Urville described two varieties under *Chrysanthemum coronarium*: var. *concolor* d'Urv., from the island of Malta, with yellow ray florets ("*radio discoque luteis sola reperitur inter segetes insulae Melitae*"), and var. *discolor* d'Urv., from the Aegean islands, with white ray florets ("*radio albo discoque luteo copiosè occurrit in insulis Archipelagi*") (d'Urville 1822).

The two entities appear to be widespread in the Mediterranean region and show no obvious correlation with geographic distribution (Turland 2004). Recently, investigation on the different population occurrence of the two varieties shown that the *discolor* variety occurs in thermo- and meso-Mediterranean areas of Greece, Italy, Spain, Portugal and the north of Morocco, whereas no occurrence of the *concolor* var. was reported in the meso-

Mediterranean environment (E. C.-O. Cano 2012) (Cano-Ortiz 2007). In the thermo-Mediterranean belt the populations of both variants tend to appear separately with only occasional concurrences (E. C. Cano 2013).

Furthermore, a kariomorphological study (El-Twab 2008) on *Chrysanthemum coronarium* varieties shown that, although the two varieties are both 2n = 18, as previously reported for *Chrysanthemum coronarium* (Bhattacharyya 1977) (Pavone 1981) (Razaq Z.A. 1988) (Nirmala A. 1986) (Vogt R. 1993) (Kaul M.L. 1995) (Strother 1997) (Carr 1999), they differ in the arrangement of two chromosomes (submedian-centromeric in *discolor* and median-centromeric in *concolor*).

In other studies, basing on the disposition of the intercostal glands, the colour of the ligules, and the size and wings of the cypselas of the flosculous flowers and their distribution, some researchers (E. C. Cano 2013) proposed to elevate the *discolor* var. to the rank of species as *Glebionis discolor* (d'Urville 1822).

Therefore, a different approach based on molecular data is needed to further investigate the genetic grounds underneath the differentiation of the two varieties.

Figure 6 Co-occurence of *Chrysanthemum coronarium concolor* and *discolor* varieties in an abandoned field (for geographic reference see Table 1 sampling ID #5)

Heterocarpy and dormancy in Asteraceae

Imbert reported that heterocarpy/heterospermy is known to occur in 18 families of angiosperms, being most common in Asteraceae and Chenopodiaceae (E. Imbert 2002). Sixty-three and three-tenths percent of the species and 52.5% of the genera belong to the Asteraceae and 8.3 and 10%, respectively, to the Chenopodiaceae. In Asteraceae, fruit heteromorphism, i.e. the difference between central and peripheral achenes (McDonough,

Germination polymorphism in Grindelia squarrosa 1975), may be associated with a difference in germination behaviour, e.g. in *Bidens*, *Senecio*, *Picris* and *Crepis* (McEvoy 1984), (Rocha 1996), (E. E. Imbert 1999), (E. Imbert 2002), (Brändel 2004), (Brändel 2007). Central achenes are often less dormant and germinate over a wider range of temperatures, to higher percentages and at faster rates than peripheral ones (J. B. Baskin 1976), (Forsyth C. 1982), (Corkid 1991) (Rocha 1996) (Brändel 2007).

In seed heteromorphism the different fruit or seed morphs usually have differential ecological behaviour, mainly germination requirements and dispersal abilities. Peripheral achenes are frequently heavier, show more limited dispersal and have stricter requirements for germination (p.e. (E. E. Imbert 1999) (Clavijo 2001). In most of the heterocarpic Asteraceae central achenes germinate immediately when favourable conditions occur while peripheral achenes show delayed germination. Differential dispersal and germination allow spreading offspring in space and time, thus reducing density dependent effects such as sib competition, predation or pathogen attack (D. H. Janzen 1971) (Augspurger 1983) (Levin S. 1984) (Harms 2000). In addition, this different ecological behaviour leads to a risk-reducing or bet-hedging strategy that is advantageous in variable and unpredictable habitats such as Mediterranean biomes and highly disturbed, man-made habitats.

Discerning the differential ecology of achene morphs in heterocarpic weedy Asteraceae is of interest in weed population dynamics and basic weed biology studies which, in turn, can be useful in designing appropriate weed management practices (Bhowmik 1997).

Seed germination

Chrysanthemum coronarium emergence occurs in autumn and seedlings develop into overwinter rosettes that bolt in January and February. Flowering and fruiting start in late February or March and continue to early summer (F. M. Bastida 2004). Flowers consist of central hermaphrodite disc florets and marginal female ray florets with inferior ovaries (Cockshull 1984). These florets develop two morphologically distinct lignified fruit called cypselae (hereafter named more merely “achenes”), which are prominently composed by sclerotic cells (Mukherjee 1992). The two morphotypes are distinguishable for their average size, peripheral much bigger than central, and for their shape: one-winged central, and three-winged peripheral achenes (Figure 7).

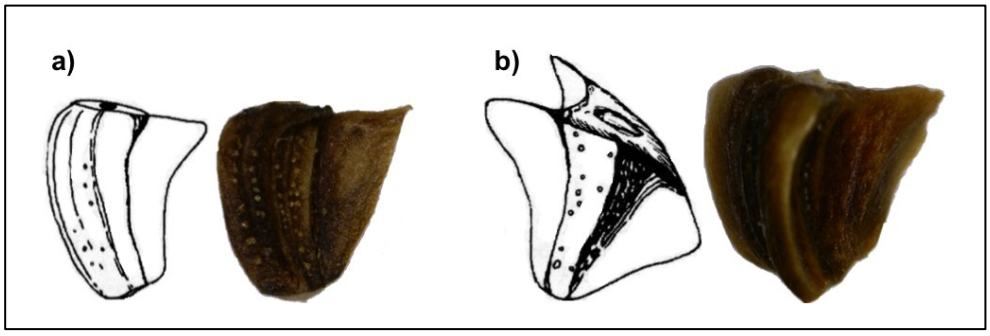

Figure 7 a) One-winged central achene; b) Three-winged peripheral achene.

Both central and peripheral achenes show dormancy in a high proportion, especially peripheral achenes are more dormant than central ones (F. M. Bastida 2004).

Even thought some studies (M. P. Chiang 1994) (Vicente 2002) (Hwang 2008) (Banon 2009) (F. J. Bastida 2010) have been investigating the germination behaviour in *Chrysanthemum coronarium*, there is still a bit of uncertainty in defining precisely the type of dormancy of this species.

In a previous study (F. M. Bastida 2004) germination was tested against different conditions: temperature regimes, dark, with a pre-chilling treatment, in presence of GA_3, of nitrate solution, and finally excising seeds from achenes. From the highest germination frequency shown in response to seed excision in presence of the pericarp tissue authors concluded that dormancy in *C. coronarium* is physically imposed by pericarp.

Conversely, in another study (Banon 2009) authors tested *C. coronarium* germinability in presence of several commercial gibberellins and excising seeds from achenes. Interestingly, when excised seeds were irrigated with pericarp leachate this produced an inhibition of germinability (from 92% to 43%), so they concluded that dormancy lies on a combination of physical and chemical dormancy.

Therefore, even though this plant is very common in Mediterranean basin, invasive outside its native environment and produces a large number of seeds, the germination behaviour remains worthy of study.

Leucanthemum vulgare

Species description and distribution

Leucanthemum vulgare (Vaill.) Lam. (family Asteaceae; tribe Anthemideae; genus *Leucanthemum*) is a monocarpic perennial herb 20 - 40 (100) cm high, with a lignified

base, hairless or hairy, simple or branched with a wiggling root-stock where new sprouts originate; flower heads are made up of 15 – 30 white rays that circle a yellow button, depressed center; florets are clustered in composite heads 2-5 cm wide, with white ray florets radiating out from a yellow centre of disk florets (Pignatti 1982) (Douglas 1998) (Jauzein 2011). His chorological type is Eurasiatic, Euri-Mediterranean and Euro-Siberian (Pignatti 1982) (Lauber 2001) (Meusel 1965). In northern Europe flowers bloom from June to August.

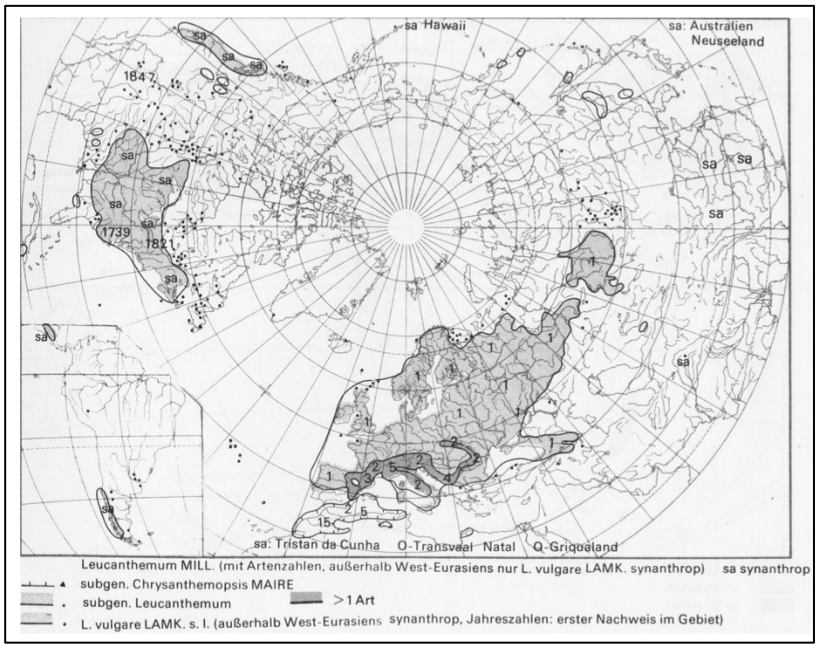

Figure 8 *Leucanthemum vulgare* distribution according to (Meusel 1965).

Leucanthemum vulgare is highly adaptable to a variety of sites. It can grow in course to medium textured soils and can be found in moist to moderately dry sites; however, it does prefer abundant sunlight. Once planted as an ornamental, it escaped cultivation and is now common in native meadows, pastures, fields in open and thick woodlands, along waterways and roadsides and it was reported as invasive plants Figure 10 (Khuroo 2010). It is also found in disturbed areas, hay fields, gardens and lawns, and irrigation ditches.

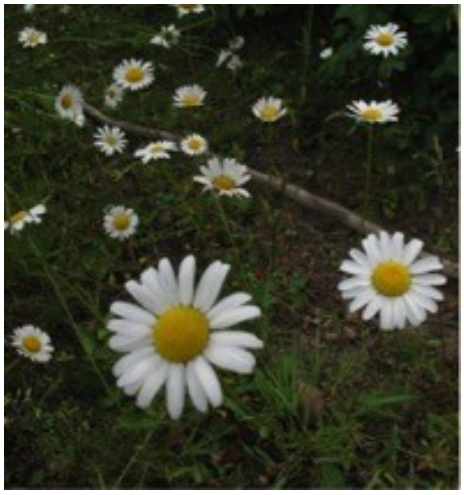

Figure 9 ***Leucanthemum vulgare***

Figure 10 A Conifer forest-opening invaded by ***Leucanthemum vulgare***

Seed germination

The fruit of *Leucanthemum vulgare* is a small flat seed (Figure 11), dark gray in color with no pappus. One plant can produce over 500 seeds and seeds can remain viable in the seedbank for up to three years.

This plant does not show any dormant behaviour and has a germination temperature between 15 and 20° C and it may germinate either in spring or in early autumn (Keller 1999). Seed germination is inhibited by continuous darkness but otherwise not affected by variation in light (K. R. Thompson 1989).

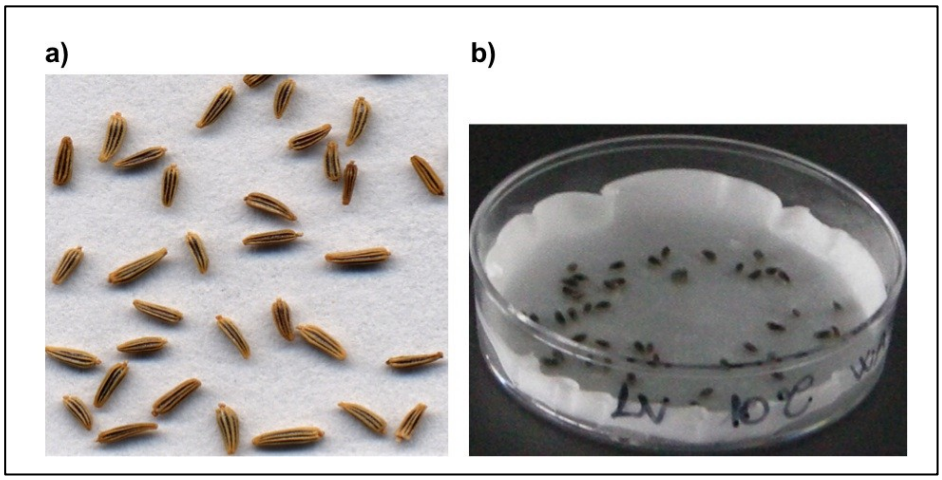

Figure 11 a) *Leucanthemum vulgare* seeds; b) *Leucanthemum vulgare* seeds imbibed in a Petri dish used for germination tests

Varietal genotyping

The assessing of genotypic diversity of closely related taxonomic entities is a challenging goal and its difficulty mainly relies on the reproductive system and species distribution.

Chrysanthemum sensu lato consisted of 27 genera which has been taxonomically placed in the subtribe Chrysantheminae O. Hoffm., the tribe Anthemideae Cass (Shih 1983) while it has been placed in different subtribes in the tribe Anthemideae (Bremer 1993). The main centers of distribution of *Chrysanthemum sensu lato* are two regions; one in the Mediterranean area and the other in China and Japan, which are considered as ancestors of the Mediterranean species (Dowrick 1952).

The varietal genotyping in *Chrysanthemum* with traditional techniques markers such as morphological and cytological markers is not effective (Wolff 1994). Therefore, other techniques for identifying molecular markers such as random amplified polymorphic DNA (RAPD) have been developed and applied (Williams 1990). The RAPD technique is more straightforward compared to RFLP analysis and requires only nanograms of genomic DNA. It has been employed in different Brassica species for varietal identification and genetic diversity studies (Jain 1994). However, this technique is sensitive to subtle changes in reaction conditions and hence difficult to reproduce.

Inter simple sequence repeat (ISSR) markers with low cost and low labour requirement but with high reliability have been developed since 1994 (Zietkiewicz E. 1994). Like any other PCR-based marker, it is rapid and require only small amount of the template DNA. ISSR amplification does not require genome sequence information but produce highly polymorphic patterns, and it seem to have the reproducibility of SSR's and the usefulness of RAPD's (Bornet 2001), and thus combine the advantages of SSR and the utility of RAPD. ISSR markers have been used to determine the genetic diversity (Qian 2001). It was also applied to the study of genetic relationships and phylogenetic analysis of Anthemideae tribe (El-Twab A. M. H. 2010).

On the other hand, DNA barcoding is a diagnostic technique for species identification, using a short, standardized DNA region, i.e., the ''DNA barcode'' (www.barcoding.si.edu) (Lahaye 2008). A DNA barcoding to properly work the sequence variation must be high enough between species so that they can be discriminated from one another; however, it must be low enough within species that a clear threshold between intra- and interspecific genetic variations can be defined. Although the use of DNA

barcoding for identification and taxonomy has been controversial (Ebach 2005) (Will 2005) a growing scientific community has embraced DNA barcoding as a practical tool for biodiversity studies, for example to facilitate inventories of very diverse but taxonomically poorly known regions (Blaxter 2004) (D. H. Janzen 2005).

DNA barcoding in plants is now better established (Hollingsworth 2009) then in the past (Rubinoff 2006) (Pennisi 2007) after the creation of the stable Plant Working Group (http://www.barcoding.si.edu/plant_working_group.html). This consortium approved the usage of *matK* and *rbcL* as the BARCODE regions for Land Plants. So that nowadays, plant barcode resources are catalogued in the Barcode Of Life Database (BOLD) (http://www.boldsystems.org/) web portal and available for search for species identification.

Aim of the thesis

The aim of this work was to perform a comparative study on two common Asteraceae weeds, a dormant species, *Chrysanthemum coronarium* var. *concolor* and *discolor*, and a non-dormant one, *Leucanthemum vulgare*, where a secondary dormancy could be inducted. In particular, the focus of this research was to determine their germination physiology, and investigating their dormancy release and dormancy induction requirements. Beyond the characterization of germination and dormancy patterns, this study aims to analyse these processes with a molecular approach through isolation of candidate genes associated with dormancy onset and its regulation.

Materials and Methods

Study sampling

Leucanthemum vulgare accession was collected in summer 2010 by Millennium Seed Bank collector team (Figure 12 and Table 1). Seeds were then cleaned and kept at +5° C with controlled RH conditions.

For *Chrysanthemum coronarium* two different seed lots were used in this study: a seed lot harvested in early summer of 2012 and another one harvested in the subsequent year in the same period, see Table 1 and Figure 12. In the older collection only *Chrysanthemum coronarium* var. *concolor* was sampled, while in 2013 field sampling *concolor* and *discolor* varieties were collected.

In 2012 sampling achenes were collected from around 20 healthy plants in a pure ample *concolor* meadow. On the other hand, in the 2013 sampling since the paucity of *discolor* plants, the harvesting was limited to 16 individuals which was the maximum number of *discolor* occurences. *Discolor* var. was always found together with *concolor* variety. During the early spring 2013 thirty-two flowering plants, sixteen individuals for each variety, were identified and labelled. From each individual an adequate amount of fresh leaves was taken and kept on ice until stored at -80° C for subsequent genotyping analyses.

Plants labelling was carried out for subsequent discrimination during seed dispersal season when is not possible to distinguish the two *C. coronarium* varieties from florets colour. Achenes of the two varieties were collected from marked plants and kept at 15% of Relative Humidity and 15° C before used in germination tests.

Collection ID	Year of collection	Taxon	Height above sea level	City - Country	Latitude	Longitude
1	2010	*Leucanthemum vulgare*	240 m	Ardingly -England	51° 3'58.40"N	0° 5'41.24"O
2	2012	*Chrysanthemum coronarium var. concolor*	110 m	Catania – Italy	37°29'25.75"N	14°59'23.63"E
3	2013	*Chrysanthemum coronarium var. concolor*	529 m	Catania – Italy	37°35'11.79"N	14°59'11.68"E
4	2013	*Chrysanthemum coronarium var. concolor*	297 m	Catania – Italy	37°33'18.86"N	14°55'52.88"E
5	2013	*Chrysanthemum coronarium var. concolor*	252 m	Catania – Italy	37°32'49.65"N	14°56'45.60"E
6	2013	*Chrysanthemum coronarium var. concolor*	320 m	Catania – Italy	37°33'41.28"N	14°58'21.60"E
3	2013	*Chrysanthemum coronarium var. discolor*	529m	Catania – Italy	37°35'11.79"N	14°59'11.68"E
4	2013	*Chrysanthemum coronarium var. discolor*	297 m	Catania – Italy	37°33'18.86"N	14°55'52.88"E
5	2013	*Chrysanthemum coronarium var. discolor*	252 m	Catania – Italy	37°32'49.65"N	14°56'45.60"E
6	2013	*Chrysanthemum coronarium var. discolor*	320 m	Catania – Italy	37°33'41.28"N	14°58'21.60"E

Table 1 GPS coordinates of sampling sites.

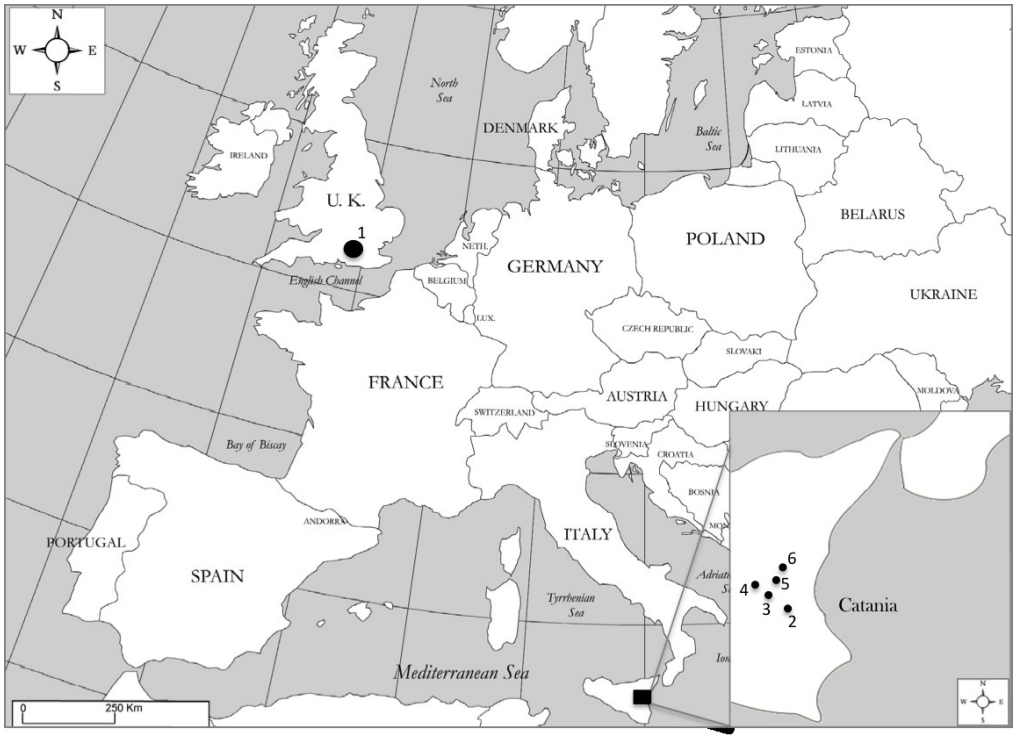

Figure 12 Map of collection of *Leucanthemum vulgare* and *Chrysanthemum coronarium*

Germination experiments

Germination experiments consisted of three replica samples of 50 seeds each. Basing on the different samples size seeds (*L. vulgare*) and achenes (*C. coronarium*) were sowed in two layers of moist filter paper (Whatmann) in plastic Petri dishes, respectively, with 5 cm diameter and with 9 cm diameter. For *Chrysanthemum coronarium* the two morphotypes were always tested separately. Filter paper imbibition was carried out always with double-distilled water when not differently indicated. Since the vast mould proliferation during germination tests, achenes of *Chrysanthemum coronarium* were always washed with 1% of commercial chlorine and then rinsed 3 times in distilled water before sowing them in Petri dishes.

Experiments were conducted in temperature (±1 ° C) and light (40 µmol m^{-2} s^{-1}) controlled conditions using a 12 h or 8/16 h daily thermo- (for alternating temperatures) and photo-period (= light hereafter). In the incubators actual temperature were checked repeatedly. Germination was defined as radicle emergence from the testa by at least 1 mm often with a small radicle flexing. All emerging seedlings were counted and removed from the dish. At the end of the experiments (upon full germination, or after 2 weeks without any further

germination) cut tests determined the number of un-germinated but viable seeds; defective seeds (i.e. empty, damaged and infected) were excluded from the all calculations.
In *Chrysanthemum coronarium* seed germination was observed to occur always after the fruit coat rupture, as a preparatory event that precedes germination, hence, FCR was recorded and used for subsequent data elaborations.
For climatic data of the collecting sites and to choose adequately the germination temperatures to use World Climate (http://www.climate-charts.com) was used as climatic reference database.

Seed excision

To observe the germination behaviour in *C. coronarium* without pericarp tissue, seeds were excised, using a razor blade, from both peripheral and central achenes and sowed at 5, 10, 15, 20, and 25° C with 12h light. For this test both seeds lots were used (2012 and 2013) to compare seeds viability. Once deduced the optimum temperature range, germination of excised seed was tested in alongside pericarp tissue to observe any possible chemical inhibitory effect.

Water uptake

In order to test the presence of physical dormancy in *C. coronarium* water uptake of intact achenes was monitored. Achenes were, hence, imbibed at 10° C and weighed with a 4-place precision balance at regular intervals of time until reaching a stable plateau. Isolated seeds were also included in this test for comparison.

Anatomical analyses of pericarp tissue

To observe the anatomical structures in the *Chrysanthemum coronarium* pericarp a tissue dissection was carried out in both achene morphotypes by using a razor blade and visualizing tissues with a microscope (Primo Star - Carl Zeiss Microscopy) using different magnifications (10X, 40X and 100X).

Germination in dark

In order to test whether *Chrysanthemum coronarium* germination is dependent on the presence of light or not, achenes from 2013 collection were tested in continuous darkness at 5, 10, 15, 20 and 25° C for 12 weeks in the dark and checked once at the end of the test. Darkness was achieved by wrapping Petri dishes in 2 aluminium foil layers and kept inside

a thick-textured black bag for the duration of the experiment. In the meantime a control experiment was carried out in light presence.

Cold stratification

A chilling stratification to mimic natural conditions during winter was carried out in *Chrysanthemum coronarium* in order to observe its impact in dormancy release. Cold stratification was carried out sowing achenes for 0, 4, 8 and 12 weeks at 5° C in darkness (see germination in darkness for methodology), and transferring them at 20, 25, 30, and 35° C after stratification.

Dry storage

Chrysanthemum coronarium central achenes from the 2012 collection were used for a long-term dry storage test that was carried out keeping achenes at 10-22° C with a Relative Humidity between 20 and 30% up to 500 days. Achenes germination were tested after 80, 200, 260 and 500d and compared with seed germination of fresh achenes.

Dry after ripening

Achenes from the 2013 collection of *Chrysanthemum coronarium* were used to test the effect of dry after-ripening treatment on seed dormancy. Hot and dry native environment summer climatic conditions were simulate keeping achenes for 0, 4, and 12 weeks at alternating temperature of 35°/20° C (night/day) with a 8/16 photoperiod with a Relative Humidity of, respectively, 35% and 58%. Afterwards achenes were imbibed at 5, 10, 15 and 20° C.

Wet warm stratification

Wet warm stratification was tested in achenes from the 2013 collection of *Chrysanthemum coronarium* to analyse the different germination response in comparison with cold stratification. Thus, achenes were imbibed at 35/20° C with 8/16 hours of photoperiod. This treatment was performed for 3, 5 or 8 weeks and after which achenes were transferred to germination temperatures 5, 10, 15 and 20° C.

Seed osmo-priming

To determine the effect on germination behaviour of seed osmo-priming treatment in *Leucanthemum vulgare* seeds were primed imbibing them with Polyethylene-glycol (PEG)

(concentration and to have an osmotic potential of -0.072 MPa) and incubated at 5° C for 1, 5, and 9 weeks. Osmo-priming at 10° C were carried out with an osmotic potential of -0.069 MPa for 1, 5, and 9 weeks. Light role in affecting germination response during priming was also tested imbibing seeds in continuous darkness for 1 week. Both for light and darkness experiments, after priming treatment seeds were washed with tap water and imbibed in double distilled water at 10, 15, 20, 25, and 30° C.

Seed hydro-priming

Leucanthemum vulgare seeds were hydro-primed by soaking seeds in water or with different osmotic potentials: -0.02, -0.072, -0.292 MPa. Afterwards seeds were washed with tap water and imbibed in double distilled water at 10, 15, 20, 25, and 30° C.

ABA synthesis

In both *Chrysanthemum coronarium* and *Leucanthemum vulgare* the ABA biosynthesis was investigated to observe its role in dormancy maintenance. To do that seeds (*L. vulgare*) or achenes (*C. coronarium*) were imbibed with different concentration 0.03, 0.1, 0.3, 1, 3, 10, 30, 50, and 100µM of fluridone (1-methyl-3-phenyl-5-[3-(trifluoromethyl)phenyl]-4(1*H*)-pyridinone) which inhibits the carotenoid pathway depleting the ABA biosynthesis (Lem 1981).

Incubation in agitated water

In *Chrysanthemum coronarium* achenes were washed in 200 ml water for 24 h on a rotating shaker to remove endogenous ABA as published in (Le Page-Degivry 1990). Afterwards, soaked achenes were used to run simultaneously two different tests: *i*) achenes were imbibed in double distillied water and incubated at 20° C; *ii*) achenes were imbibed with a 100µM solution of fluridone and incubated at 20° C. In the first test, water washing was done to remove the ABA already present in the achene, while in the second test, in addition to removing the pre-existing ABA, fluridone was added to prevent any further ABA biosynthesis during the achene imbibition.

Giberellic acid

After the osmo-priming treatment, *Leucanthemum vulgare* seeds appeared to be inducted in a non-deep physiological dormancy status. Thus, in order to investigate the degree of physiological dormancy, osmo-primed seeds were imbibed with giberellic acid at different

concentrations 0.01, 0.03, 0.1, 0.3, 1, 3, and 10 µM. control experiment was carried out by imbibing seeds in double distilled water.

Molecular analyses

Chrysanthemum coronarium varietal genotyping

To investigate the genetic relationship between the two varieties of *Chrysanthemum coronarium,* two different approaches were carried out: an intra-specific analysis using markers which produce highly polymorphic patterns to evaluate their genetic diversity; an inter-specific approach to investigate whether the genetic diversity degree arises from differences at the species rank or not. In the first system the profiles of Inter Simple Sequence Repeats (ISSRs) were used, and in the second one ribulose-bisphosphate carboxylase gene (rbcL) was sequenced on both strands.

DNA extraction

For each of the 32 individuals (16 *concolor* + 16 *discolor* var.) around 90 mg of fresh leaf tissue was used to extract DNA following the CTAB/Chloroform-Isoamyl alcohol protocol published by (J. J. Doyle 1987) (J. J. Doyle 1987) (Cullings 1992).

Even though the presence of 4% Polyvinylpyrrolidone (PVP) in the extraction buffer the polyphenolic compounds exerted an inhibitory effect on the Taq polymerase activity during the amplification DNA. Thus, a final concentration of 2% of Polyvinylpyrrolidone was added to the PCR mix (Koonjul 1999) to have reproducible and stable DNA amplifications. From DNA extraction 25 ng of DNA was used as template for subsequent PCR analyses.

ISSR profiling

Internal Sequence Simple Repeats (ISSR) PCR were carried out for all the samples in a total volume of 25 µL containing 25 ng of DNA template, 2.5 µL of 10 × PCR buffer (100 mM Tris–HCl, 15 mM $MgCl_2$, 500 mM KCl), 2% of PVP, 0.5 µL of dNTPs (10 mM each), 0.5 µL of primer (10 mM), 1.5 unit of Solis HotStart Taq DNA polymerase (Solis BioDyne, Tartu, Estonia).

Twenty-one ISSR markers, 3’ or 5’ anchored primers (synthesized by Eurofins MWG Operon, Ebersberg, Germany), were tested and 9 of them were selected basing on their band pattern and reproducibility (see Table 2).

Primer ID	Primer sequence 5'-3'	Selected for genotyping	Annealing temp. (° C)	Source of publication
Cc-01	ACCACCACCACCACCACCG	Y	48	(Dogan 2007)
Cc-02	GAGAGAGAGAGAGAGAGAC	Y	48	(Dogan 2007)
Cc-03	AGAGAGAGAGAGAGAGAGC	Y	50	(Dogan 2007)
Cc-04	ACACACACACACACACACG	Y	50	(Dogan 2007)
Cc-05	ACACACACACACACACCG	Y	50	(Dogan 2007)
Cc-06	ACACACACACACACACCY	N	50	(Dogan 2007)
Cc-07	CAGCACACACACACACACA	N	50	(Dogan 2007)
Cc-08	CGTCACACACACACACACA	N	50	(Dogan 2007)
pBr_01	GAGCAACAACAACAACAA	N	50	(Huangfu 2009)
pBr_02	CTGGTGTGTGTGTGTGTGT	N	50	(Huangfu 2009)
pBr-03	AGAGAGAGAGAGAGAGGTG	Y	50	(Huangfu 2009)
pBr_04	GAGAGAGAGAGAGAGAACC	Y	50	(Huangfu 2009)
pBr_05	AGAGAGAGAGAGAGAGYC	Y	50	(Huangfu 2009)
pBr_06	CTCTCTCTCTCTCTCTRA	N	50	(Huangfu 2009)
pBr_07	DBDACACACACACACAC	N	50	(Huangfu 2009)
pBr_08	HVHTGTGTGTGTGTGTG	N	50	(Huangfu 2009)
He-816	CACACACACACACACAT	N	50	(Smissen 2003)
He-821	GTGTGTGTGTGTGTGTT	N	50	(Smissen 2003)
He-819	GTGTGTGTGTGTGTGTA	N	50	(Smissen 2003)
Hy_18	GTGCTCTCTCTCTCTCTC	N	50	(Barcaccia 2006)
Hy_39a	AGCAGCAGCAGCAC	Y	50	(Barcaccia 2006)

Table 2 Primers sequences used for varietal genotyping in *Chrysanthemum coronarium*

The amplification reactions were carried out using a Quanta biotech SI-96 Thermal Cycler (Quanta Biotech Ltd, Surrey, UK) with the following PCR profile: 95° C for 15 min; 35 cycles at 94° for 30 sec, specific annealing temperature for each primer (Table 2) for 1 min, 72° C for 1 min; and final extension at 72° C for 10 min.

Ten ul of PCR products were electrophoresed on 2.0% agarose gels during 2.0 h at 100 V in 1Å~ TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.4) and a 100 base-pair ladder (100 bp DNA Ladder, Solis BioDyne, Tartu, Estonia) was used as molecular size marker. Gel was stained for 30 min with 1X Gel Red (© Biotium, USA) and then visualized and photographed using a gel documentation system (BioDoc-It® Imaging System, CA).

rbc-L gene amplification and sequencing

The entire rbcL gene was amplified using the "1f" and "1369 r" primers as described in (Olmstead 1992) in a total volume of 70 µL containing 25 ng of DNA template, 7 µL of 10 × PCR buffer (100 mM Tris–HCl, 15 mM $MgCl_2$, 500 mM KCl), 8.4 µL of $MgCl_2$ (25

mM), 2% of PVP, 1.4 µL of dNTPs (20 mM each), 1.75 µL of primer (10 mM), 2 unit of Solis HotStart Taq DNA polymerase (Solis BioDyne, Tartu, Estonia).

Amplifications were carried out in the thermal cycler aforementioned with the following PCR profile: 95° C for 15 min; 35 cycles at 94° for 30 sec, 1 min at 48°, 72° C for 1 min; and final extension at 72° C for 10 min. The obtained PCR products were purified and sequenced on both strands by Macrogen Inc. (Netherlands) using PCR primers. Sequencing was conducted under BigDye™ terminator cycling conditions and products purified using ethanol precipitation and run using automatic sequencer ABI-3730XL.

Data analysis

ISSR Band analysis was carried out using Total Lab 100 (TotalLab Ltd, UK) and only non-overlapping and highly reproducible bands with a molecular size comprised from 350 to 1700bp were considered for fragments detection.

Genetic diversity was measured by the percentage of polymorphic bands (P), which was calculated by dividing the number of polymorphic bands at population and species levels by the total number of bands surveyed. Shannon indices of diversity, namely both the total diversity (Hsp) and the intra-population diversity (Hpop), were also calculated using the computer program POPGENE software package Version 1.32.

Sequences of rbc-L genes were blasted against BOLD databases (http://www.boldsystems.org/) (Ratnasingham 2007) and best score hits were considered for subsequent analysis. Sequence alignment was carried out using CLUSTAL W algorithm (Larkin 2007).

Identification and isolation of reference and dormancy associated genes in *C. coronarium* and *L. vulgare*

Reference genes

Quantifying gene expression levels through reverse transcription–quantitative real-time PCR (RT–qPCR) is the preferred method for targeted gene expression measurements. However, normalization, is necessary to correct for sample input and reverse transcriptase efficiency, is a crucial step to obtain reliable RT–qPCR results. Stably expressed genes (i.e. genes whose expression is not affected by the treatment or developmental stage under study) are indispensable for accurate normalization of RT–qPCR experiments. Lack of accurate normalization could affect the results and may lead to false conclusions.

Some of the house keeping genes involved in basic cellular activities such as *18s rRNA*, *25s rRNA*, *glyceraldehyde-3-phosphate dehydrogenase* (GAPDH) and *ubiquitin* (UBQ) are some of the commonly used internal control genes as they are likely to be expressed at constant levels regardless of experimental conditions (Schmittgen 2000) (Czechowski 2005).

Therefore, selected genes from literature (Dekkers 2011) were used to find their homologous gene family for Asteraceae consulting "Nucleotide" dataset of "NCBI" database. Afterwards, each matched sequences was searched at the *Arabidopsis* electronic fluorescent pictographic (eFP) browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) (Schmid 2005) (Kilian 2007) (Winter 2007) to check how much stable is the expression of the selected gene compared with the treatment as reported in the browser. Most stable gene sequences, hence, were aligned for cloning primers designing to use in downstream *C. coronarium* and *L. vulgare* PCR amplifications.

Dormancy associated genes

On the basis of the specific literature on molecular regulation of seed dormancy (Liu 2007) (G. C. Chiang 2009) (L. H.-L.-B. Bentsink 2010) (Footitt 2011) DOG1 *(DELAY OF GERMINATION 1)*, FLC *(FLOWERING LOCUS C)*, HUB2 *(HISTONE MONOUBIQUITINATION 2)* genes were included. In addition the EXP *Expansin* gene was selected for his role in mediating cell expansion during radicle elongation (F. D. Chen 2001).

The overall workflow used for marker selection is illustrated in Figure 13.

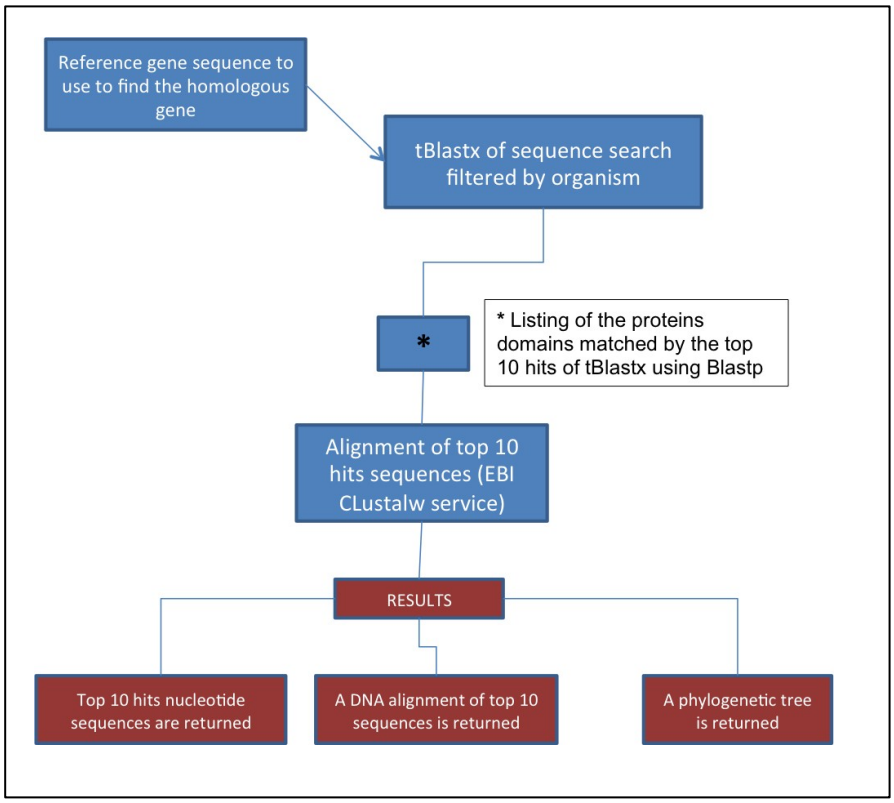

Figure 13 Workflow for primer designing of dormancy associated genes in *C. coronarium* and *L. vulgare*

DNA extraction

Fifty seedlings of *Leucanthemum vulgare* about 1 cm long each along with their seed coat were grounded in a mortar pestle using liquid nitrogen for DNA extraction. A CTAB/Chloroform-Isoamyl Alcohol procedure was used for DNA extraction as in (Kocik 1996). Samples were diluted 1000 times, shared through vigorous vortexing to make DNA more accessible for downstream amplification purposes.

Both *L. vulgare* and *C. coronarium* (herein for this chapter just *concolor* variety, DNA extracted following method described at the beginning of this chapter) extracted DNAs were used as templates for amplification of reference genes and dormancy-associated genes.

RNA extraction

Total RNA was isolate from different stage of dormancy of *L. vulgare* seeds: dry seeds (as reference control), 1 week primed seeds at 5° C, 1 week primed seeds at 10° C, 5 weeks primed seeds at 5° C, 5 weeks primed seeds at 10° C, and after imbibition upon germination (endosperm rupture and when upon radicle protrusion not longer then 1 mm). Each sample was made up by three replicas of 50 seeds each. RNA was extracted using the hot borate protocol as in (Wan 1994) (Toorop 2005). Pellets were dried over-night using a

freeze-dryer (Maxi Dry Lyo, Gemini BV) and, afterwards, resuspended in 50 µl of RNA-secure™ (Ambion®, Life Technologies) to inactivate RNases. RNA was quantified with a spectrophotometer WPA Lightwave (Biochrom, Cambridge, UK). Half µg per each sample was loaded in a electrophoresis gel well, run at 100 V in 1Å~ TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.4) for 1 hour and visualized through a Gene Genius (Syngene, Synoptics Ltd) bio imaging system.

cDNA first strand synthesis

One µg of total RNA was used as a template for first cDNA strand with Transcriptor First Strand cDNA Synthesis kit (Roche, UK), using anchored-oligo$(dT)_{18}$ primers. First strand polymerization was carried out in PCR tubes using a thermal cycler DNA Engine Dyad, Peltier Thermal Cycler. Synthesized cDNA was used as template for amplification of reference genes and dormancy-associated genes.

PCR products cloning

Three µL of PCR products were used for ligation with a TA cloning vector, pGEM®-T easy Vector kit (Promega), following the manufacturer instructions with some modifications: 1 µL of ligation reaction was used for transformation and LB medium (for 1 L: 10 g BactoTryptone, 5 g of BactoYeast, and 5 g NaCl) was used. Bacteria were grown in a 1.5 mL polypropylene tube for 1.5 h and 100 µL were plated in a LB/ampicillin plate and incubated at 37° C over-night. Single colonies were picked up with wooden sticks and rinsed with double distilled sterile water in an Eppendorff tube. Ten µL were used as template for PCR colony using plasmid specific primers to amplify the inserted DNA fragment. Electrophoretic gel runs were carried out for evaluating the size of the incorporated fragment and comparing it with the supposed gene length as observed during homologous gene alignment.

When positive colonies were found bacteria were picked up from the twin colony and grown over night at 37° C in 3 mL of LB-medium containing the ampicillin antibiotic. Isolation of plasmid DNA was carried out using the High Pure Plasmid Isolation kit (Roche) starting from a 3 mL of cell culture (transferring two times 1.5 ml to an Eppendorf tube and centrifuging at 14000 rpm for 30 sec). Isolated plasmid DNA samples were quantified using a spectrophotometer (see above for instruments specifications), checked through inserted fragment amplification, and sequenced by University College London sequencing service.

Sequences analysis

Plasmid sequences were trimmed against vector database to remove any contaminations and then blasted using BLASTn algorithm (NCBI) against Asteraceae dataset sequence or if no results were returned enlarging the dataset including more plant groups. The most similar sequences resulted in homologous genes and the most similar sequences were aligned using CLUSTAL W algorithm (Larkin 2007). This procedure gave an evidence of the origin of isolated sequences (Figure 14).

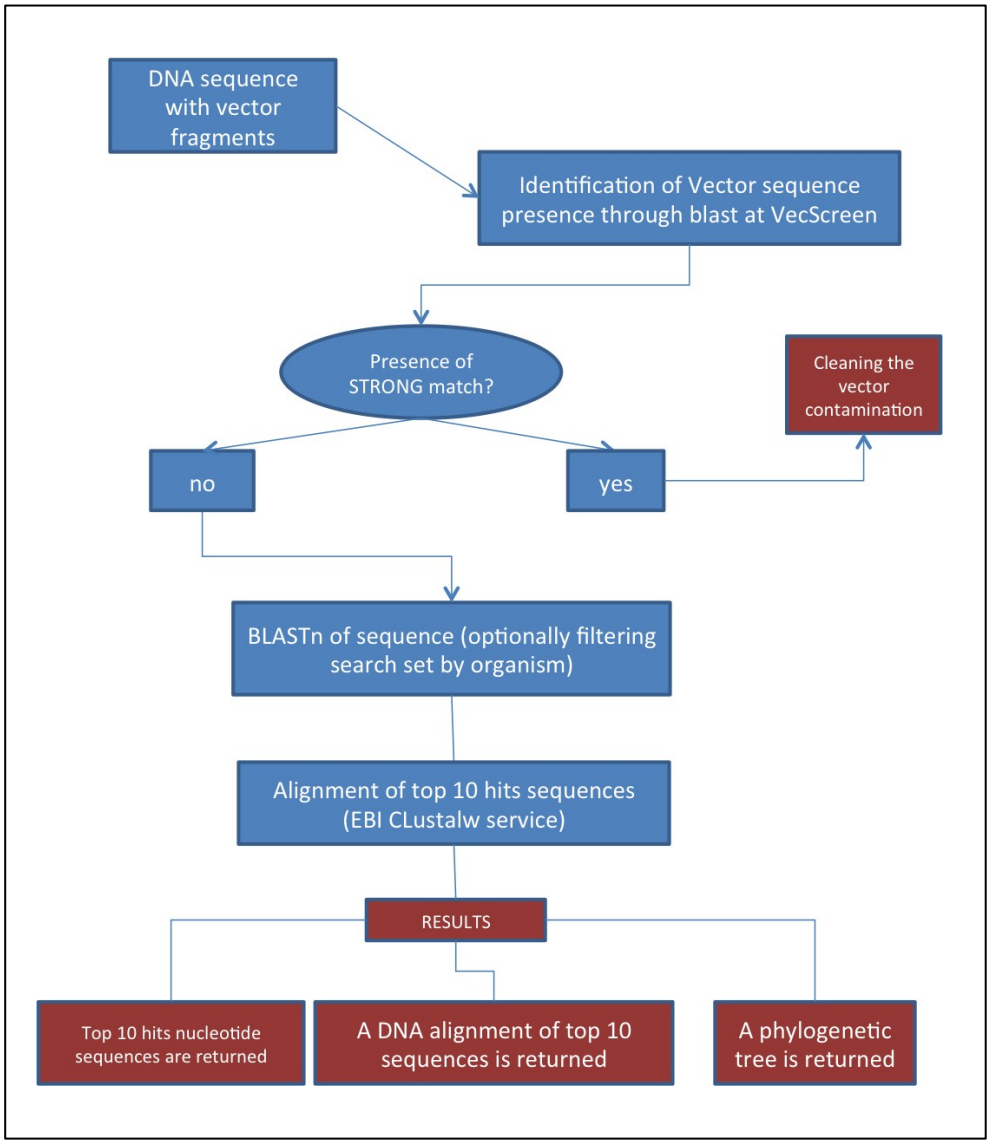

Figure 14 Sequence annotation workflow

Results

Physiology of germination of *Chrysanthemum coronarium*

Germination behaviour

Chrysanthemum coronarium germination of fresh achenes in the two varieties (see Figure 15 and Figure 16) showed a very low response (under 5 %). Radicle emergence always occurred in achenes with an evident rupture in the fruit coat, but not all the ruptured achenes germinated. Higher temperatures produced a significantly increase in FCR events then lower temperatures.

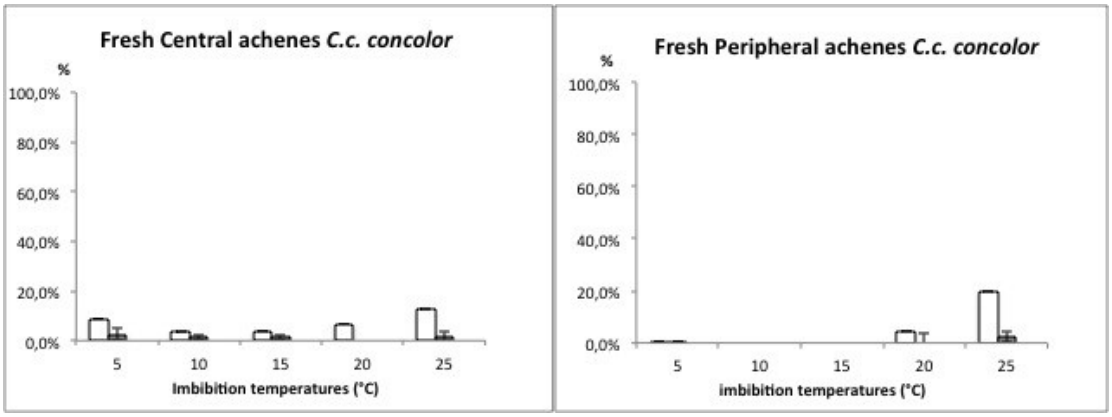

Figure 15 *Chrysanthemum coronarium* var. *concolor* germination at constant temperatures. Fruit coat rupture: open histograms; Seed germination: solid histograms.

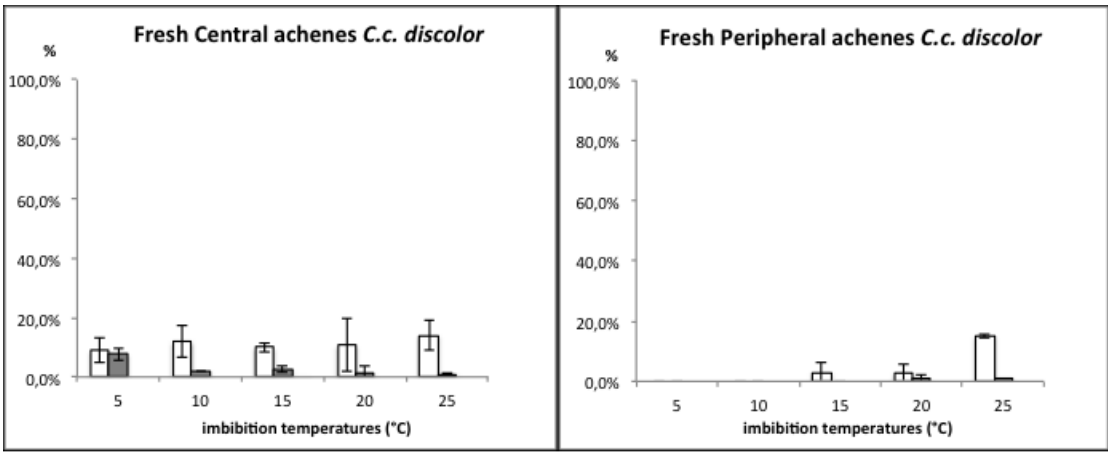

Figure 16 *Chrysanthemum coronarium* var. *discolor* germination at constant temperatures. Fruit coat rupture: open histograms; Seed germination: solid histograms.

Seed excision

Excising seeds from pericarp resulted in very high germination frequency (100%) for both peripheral and central morphotypes (Figure 17). Values on germination speed (t-50) indicated a slower response at low temperatures (Figure 17).

On the other hand, intact achenes germinated fewer than 5% in all the tested temperatures.

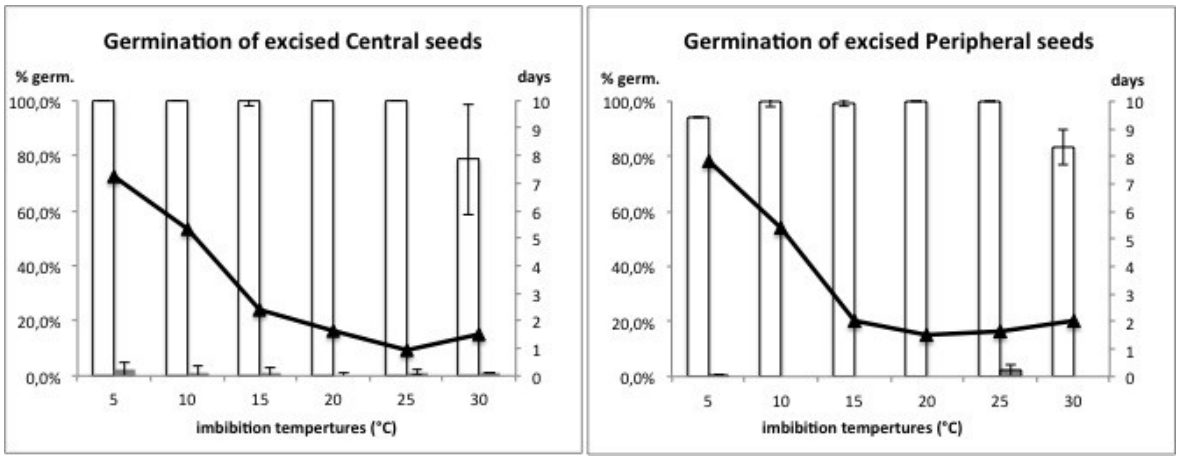

Figure 17 Germination of central and peripheral excised seeds of *Chrysanthemum coronarium* var *concolor*. Histograms follow principal axis (final germination %), while curve follows the secondary axis (number of days until 50% of final germination). Open histograms: seed germination of excised seeds. Solid histograms: seed germination of intact achenes. Solid line: excised seed germination t-50.

Furthermore, sowing excised seeds in close contact with pericarp tissue did not showed any significant inhibitory effect (Figure 18).

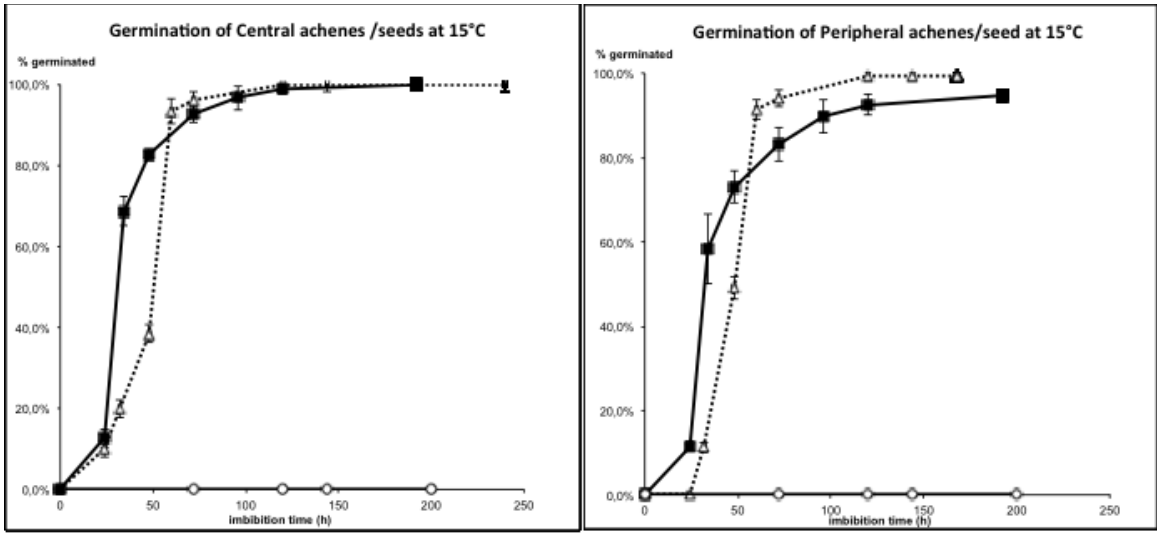

Figure 18 Germination at 15° C of central and peripheral excised seeds of *Chrysanthemum coronarium* var *concolor* in close contact with pericarp tissue; Open circle: intact achenes (control experiment); Solid line: excised seeds with pericarp tissue; Open triangles: excised seeds without pericarp tissue.

Water uptake

Central and peripheral excised seeds showed an increase of weight after imbibition in water and reached a plateau after 48h. This trend is clearly visible in imbibed intact achenes as well (Figure 19). The comparison of relative weights during imbibition showed a more pronounced increase in peripheral excised seeds weights than in central ones.

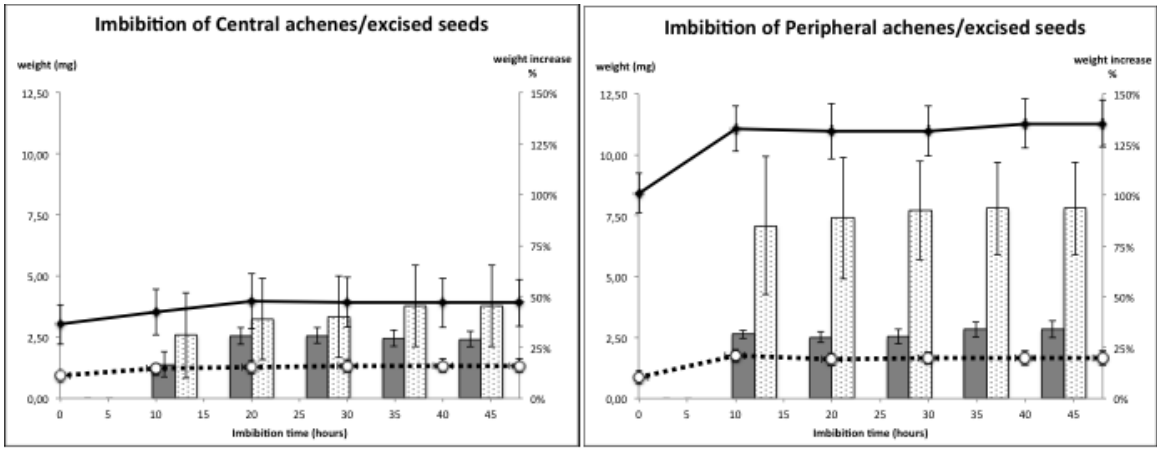

Figure 19 *Chrysanthemum coronarium* var. *concolor* seed imbibition time course. Weight (mg) in principal axis, weight increase (%) in the secondary axis. Curves follow principal axis, while histograms follow secondary axis. Intact achenes are represented by solid line and solid histograms. Excised seeds are represented by dotted lines and dotted histograms.

Anatomical analyses of pericarp tissue

Anatomical analyses conducted on pericarpic tissue in both achene morphotypes (Figure 20 and Figure 21) unveiled the presence of *brachysclereids* cells in mesocarp. The presence of various pit canals in these structures can allow water flow during achene imbibition (Figure 22).

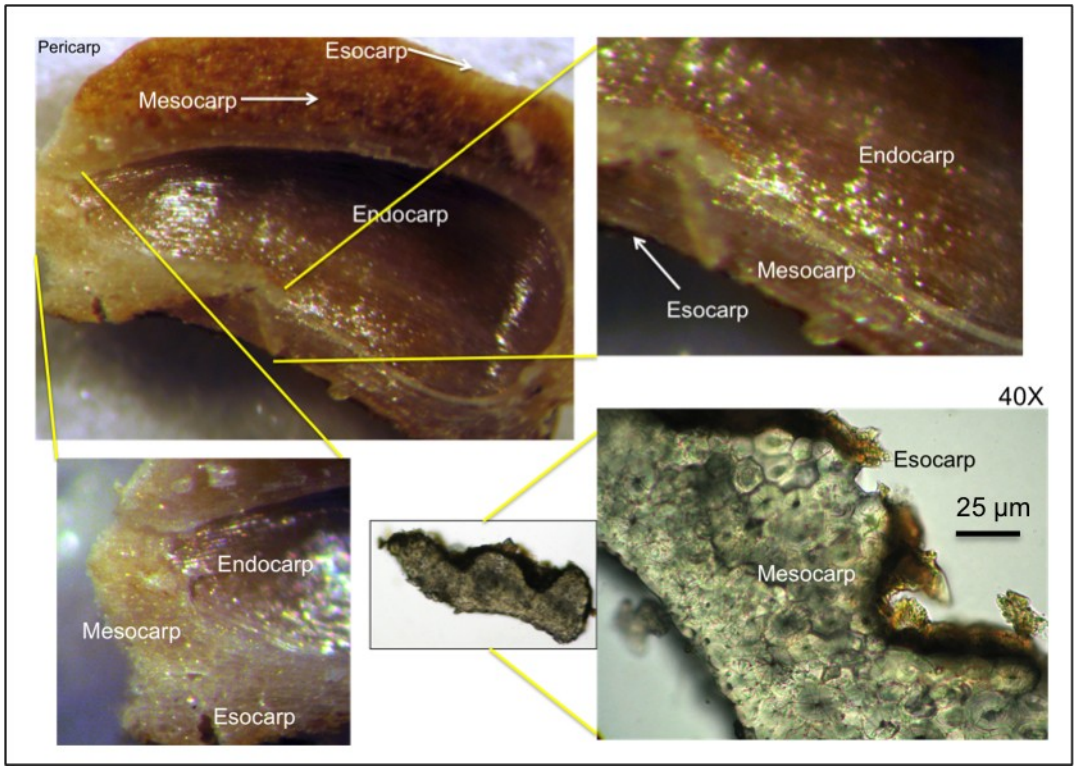

Figure 20 Anatomy of central achene pericarp. On the bottom left a 40X magnification of a cross section of pericarp where sclerotic cells with pit canals are distinguishable.

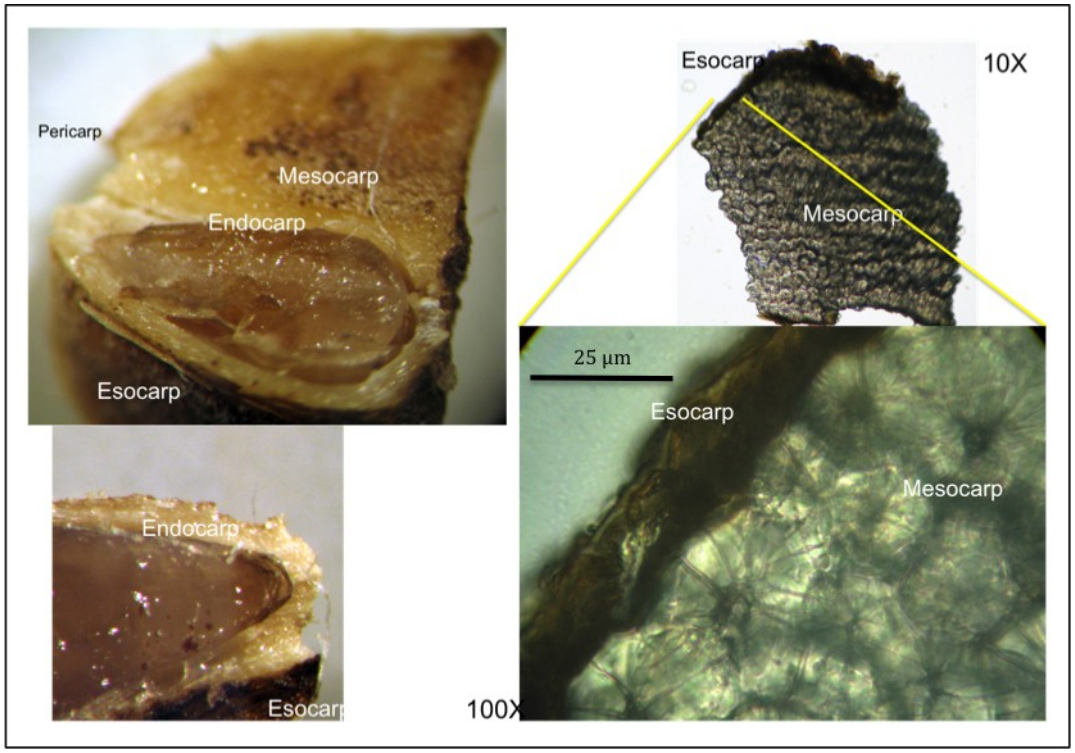

Figure 21 Anatomy of peripheral achene pericarp. On the bottom left a 100X magnification of a cross section of pericarp where sclerotic cells with pit canals are distinguishable.

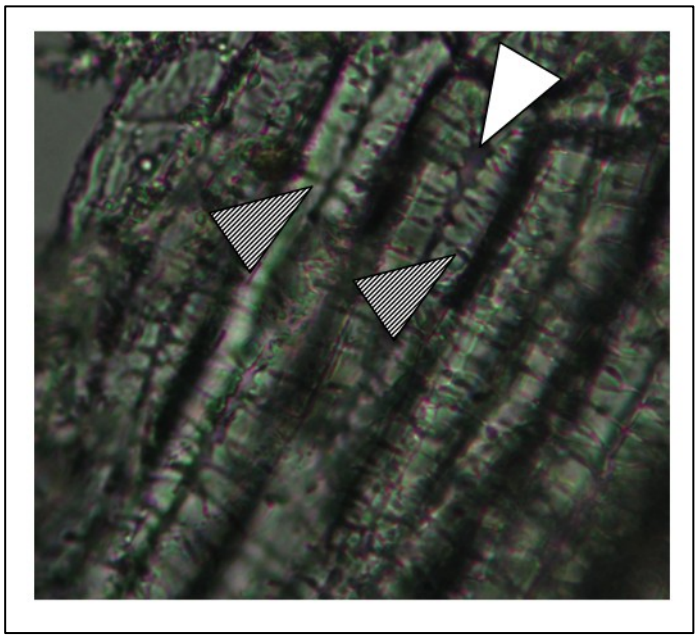

Figure 22 A 100X magnification of a longitudinal section of pericarp. Thick-walled lignified brachysclereids with cell lumen (white arrowhead) and pit canals (striped arrowhead) some of which are branched.

In the following chapters an investigation on the release of physiological dormancy is undertaken.

Germination in dark

The presence of light seems not be significant in germination promotion for both morphotypes. Beyond light or dark conditions higher temperatures results in higher FCR values.

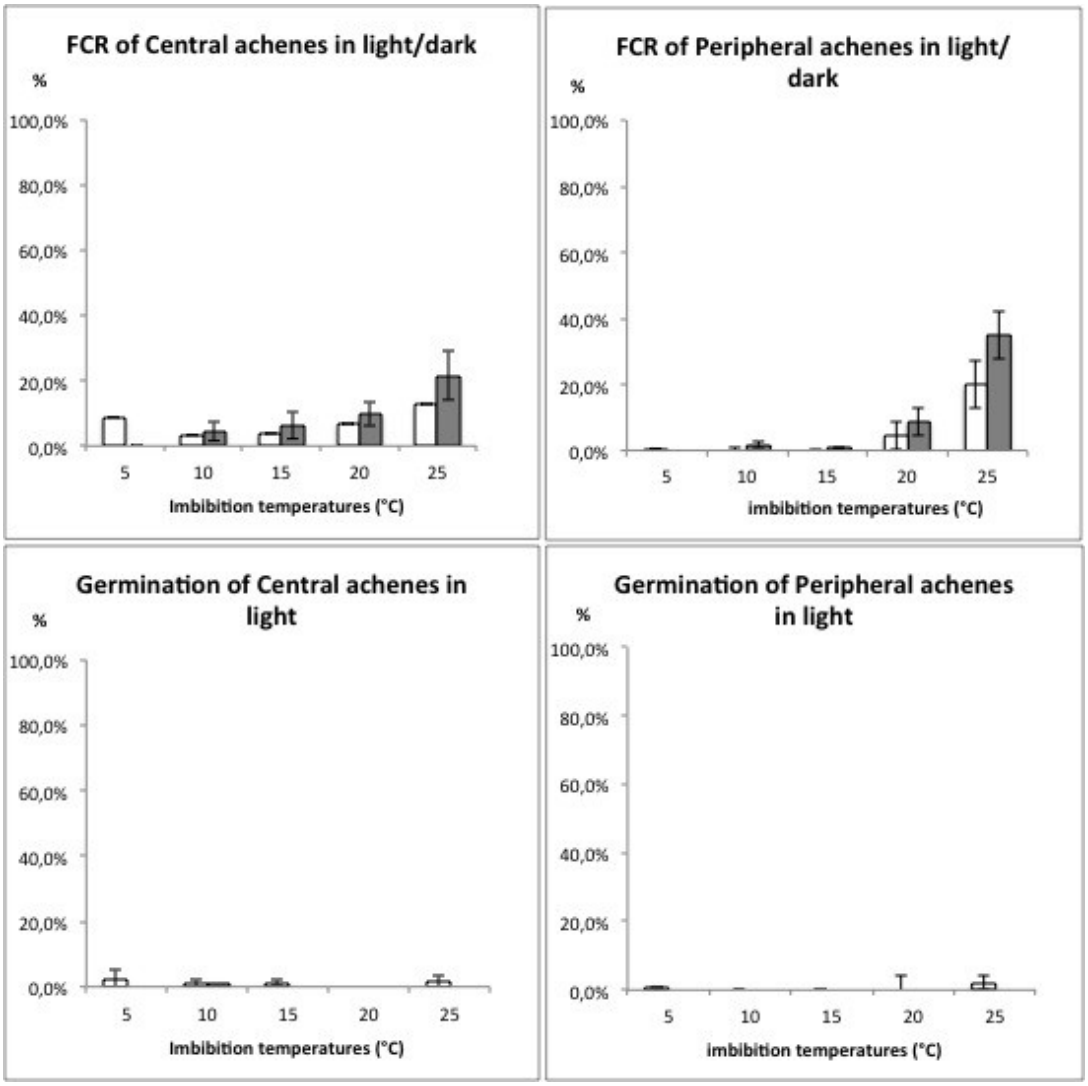

Figure 23 *Chrysanthemum coronarium* var. *concolor* germination in darkness. Fruit coat rupture on top of the figure and below germination frequency. Solid histograms: achenes imbibed in darkness; open histograms: achenes imbibed in light.

Cold stratification

For both morphotypes, at the end of cold stratification no germination neither fruit coat rupture was observed. Fruit coat rupture trend followed the temperatures gradient being more pronounced at higher temperatures with no significant variations respect to the control experiment. Increasing cold stratification time period incubation did not produced any significant growth in germination results.

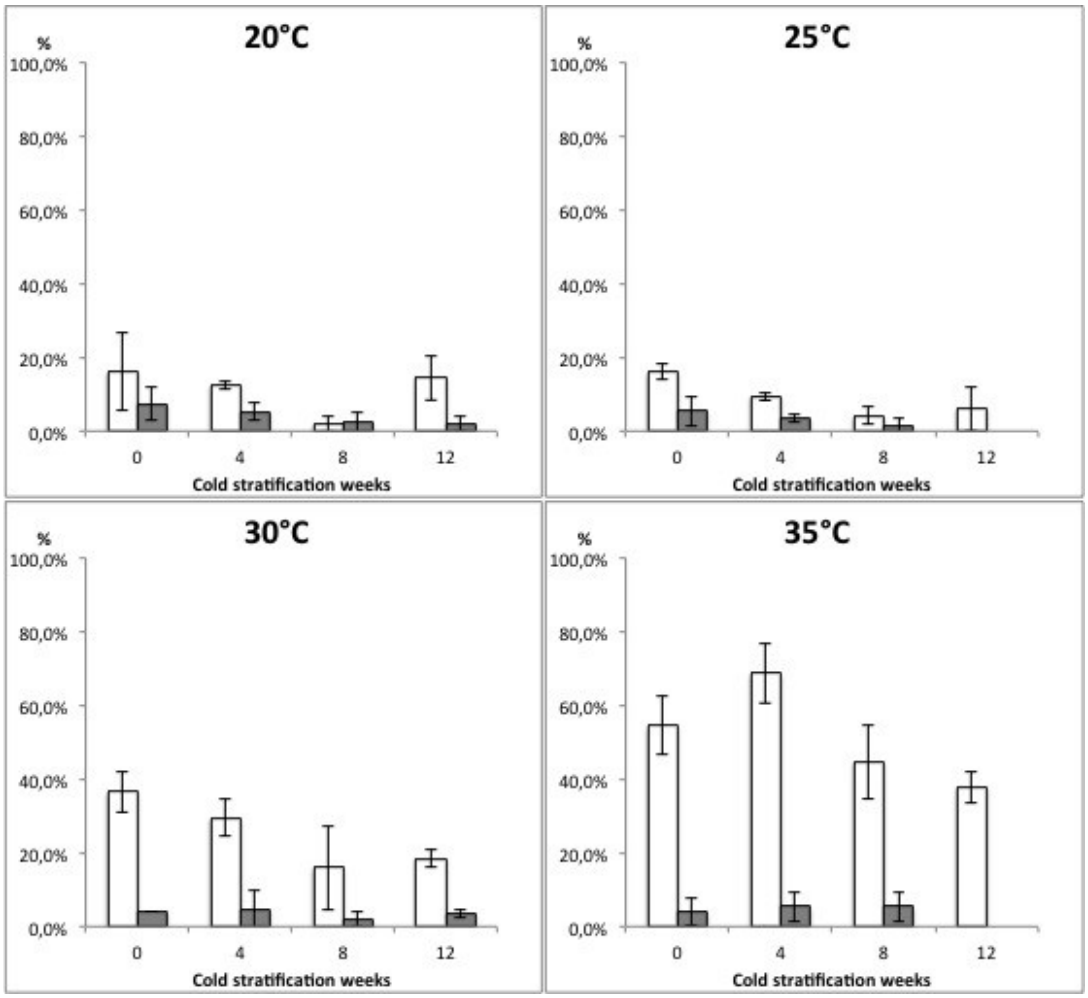

Figure 24 *Chrysanthemum coronarium* var. *concolor* central achenes were stratified at 5° C in darkness for 4, 8 or 12 weeks before imbibition at germination temperatures (20, 25, 30, and 35° C). Fruit coat rupture: open histograms; Seed germination: solid histograms.

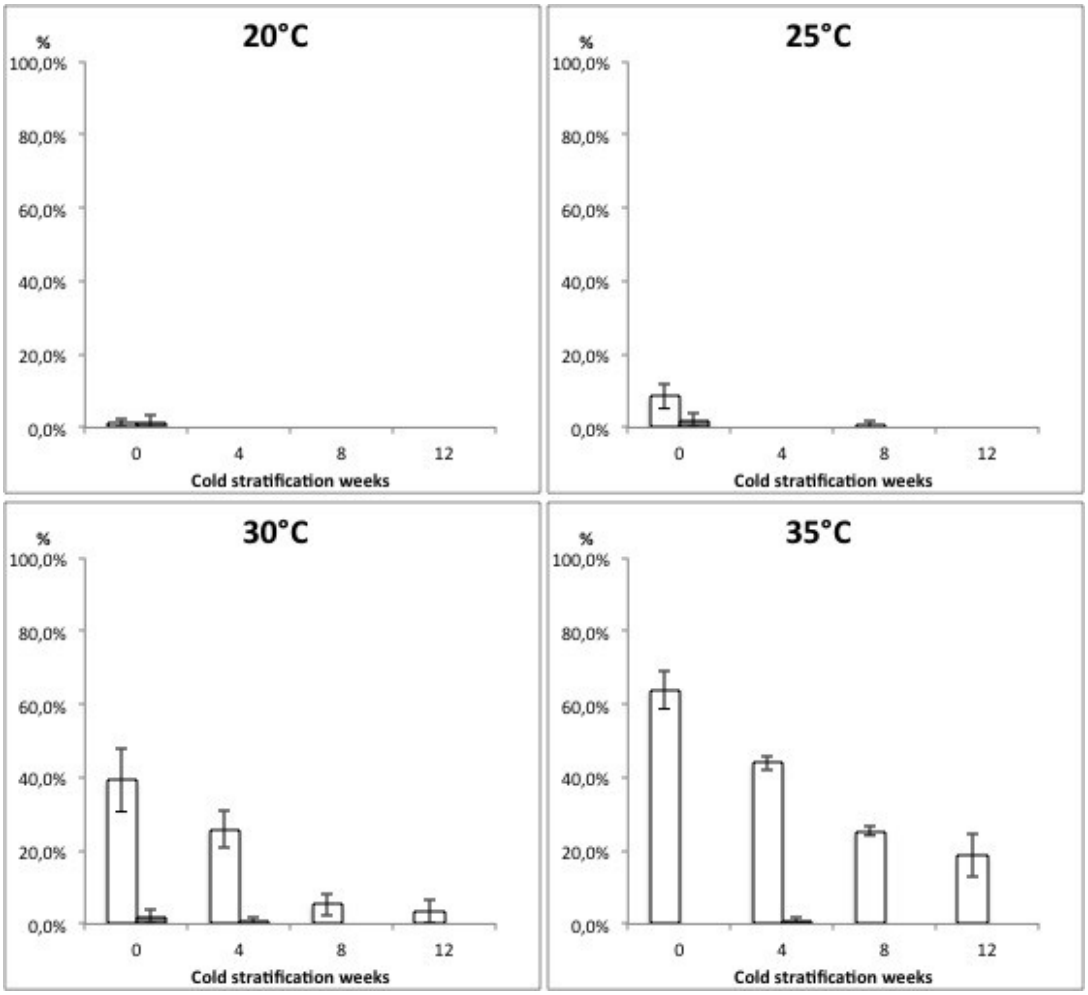

Figure 25 *Chrysanthemum coronarium* var. *concolor* peripheral achenes were stratified at 5° C in darkness for 4, 8 or 12 weeks before imbibition at germinaition temperatures (20, 25, 30, and 35° C). Fruit coat rupture: open histograms; Seed germination: solid histograms.

Dry storage

Achenes dried for 260d showed the highest germination percentage especially when imbibed at low temperatures, with a maximum germination frequency at 5° C (37,5%), Figure 26.

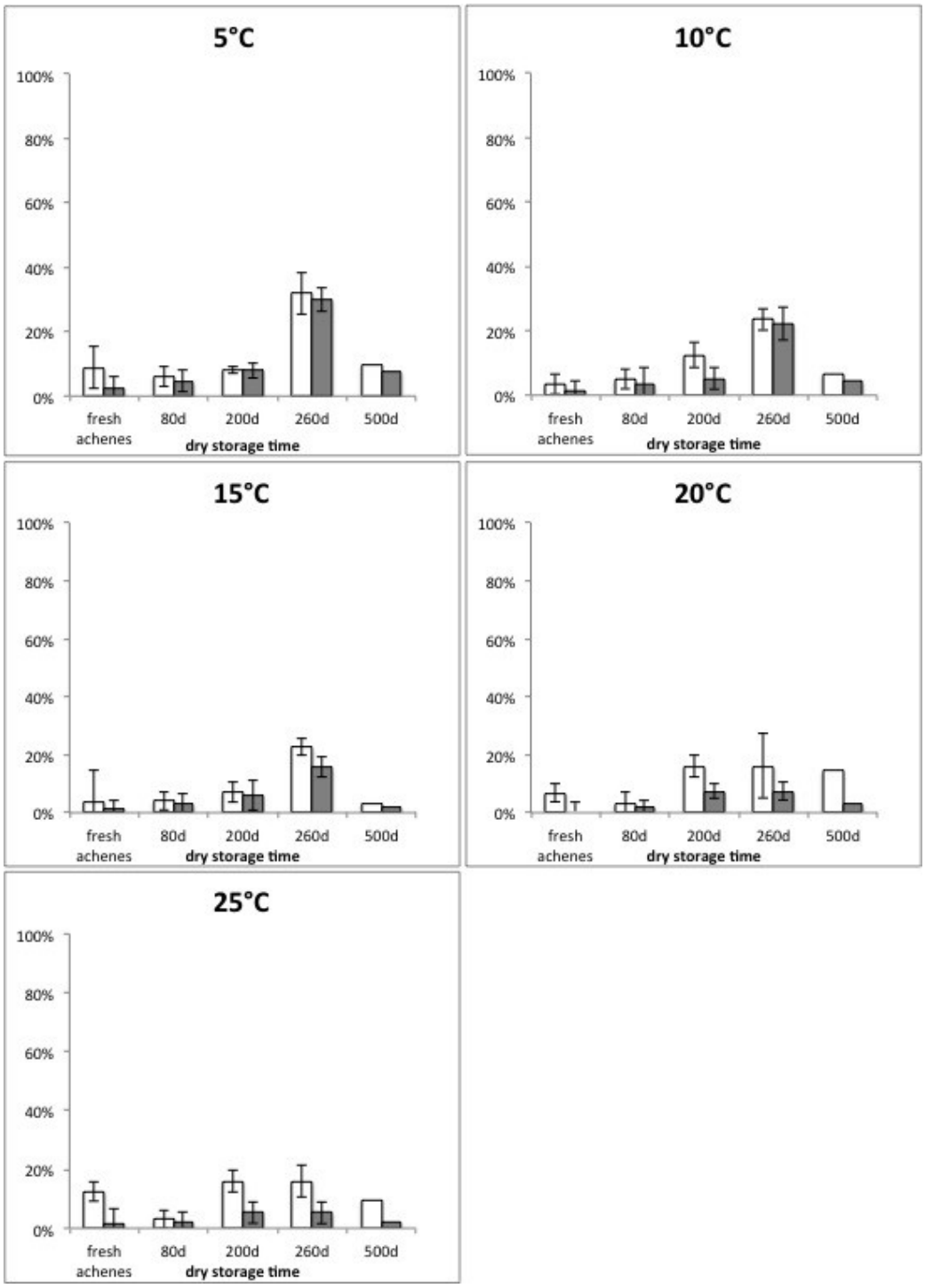

Figure 26 *Chrysanthemum coronarium* var. *concolor* central achenes dry storage for 80d, 200d and 260d. Fruit coat rupture: open histograms; Seed germination: solid histograms.

Dry after ripening

From the dry after-ripening treatment achene germination did not showed significant increase (always lower than 12%) respect to control experiment with fresh achenes.

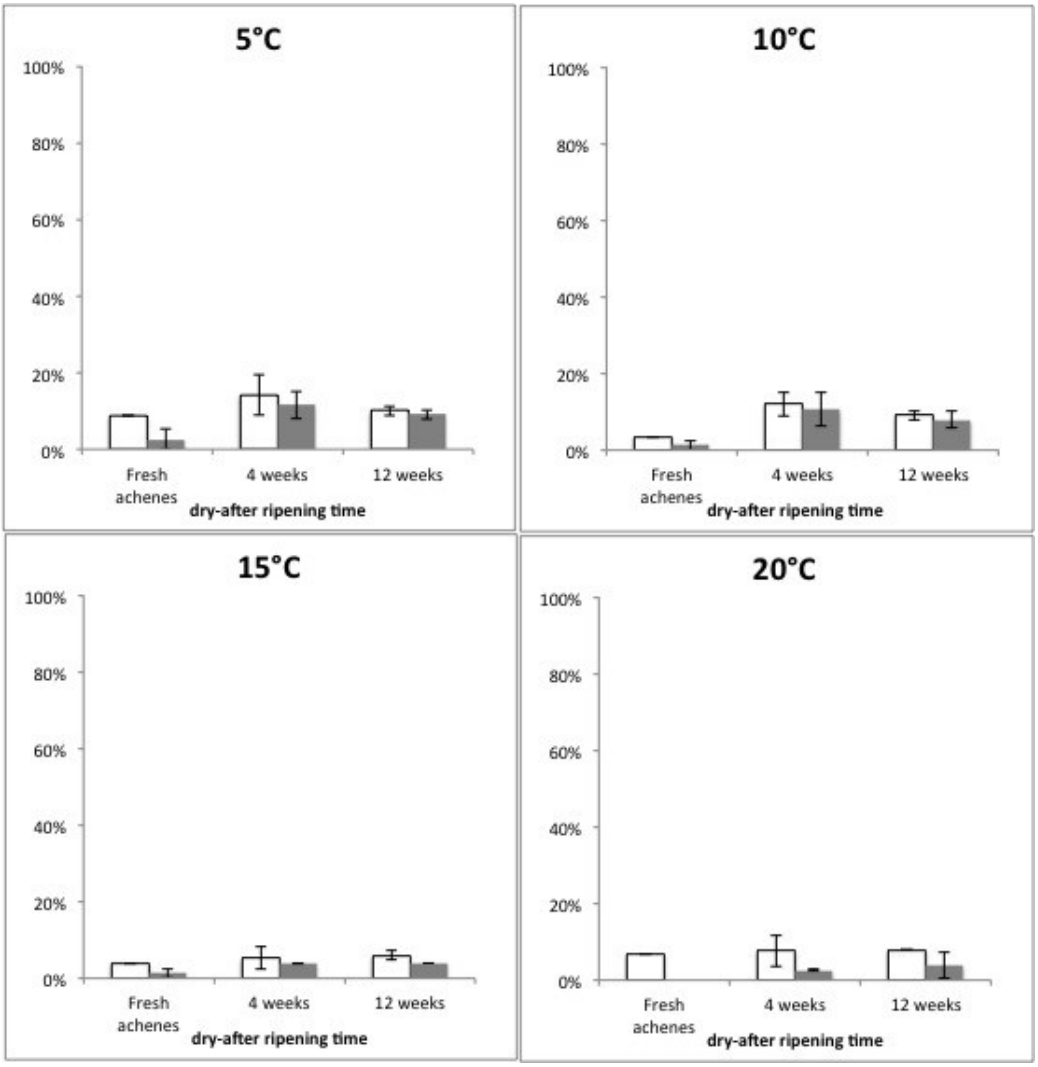

Figure 27 *Chrysanthemum coronarium* var. concolor central achenes dryied after-ripening with alternating temperatures of 35/20° C (night/day). Fruit coat rupture: open histograms; Seed germination: solid histograms.

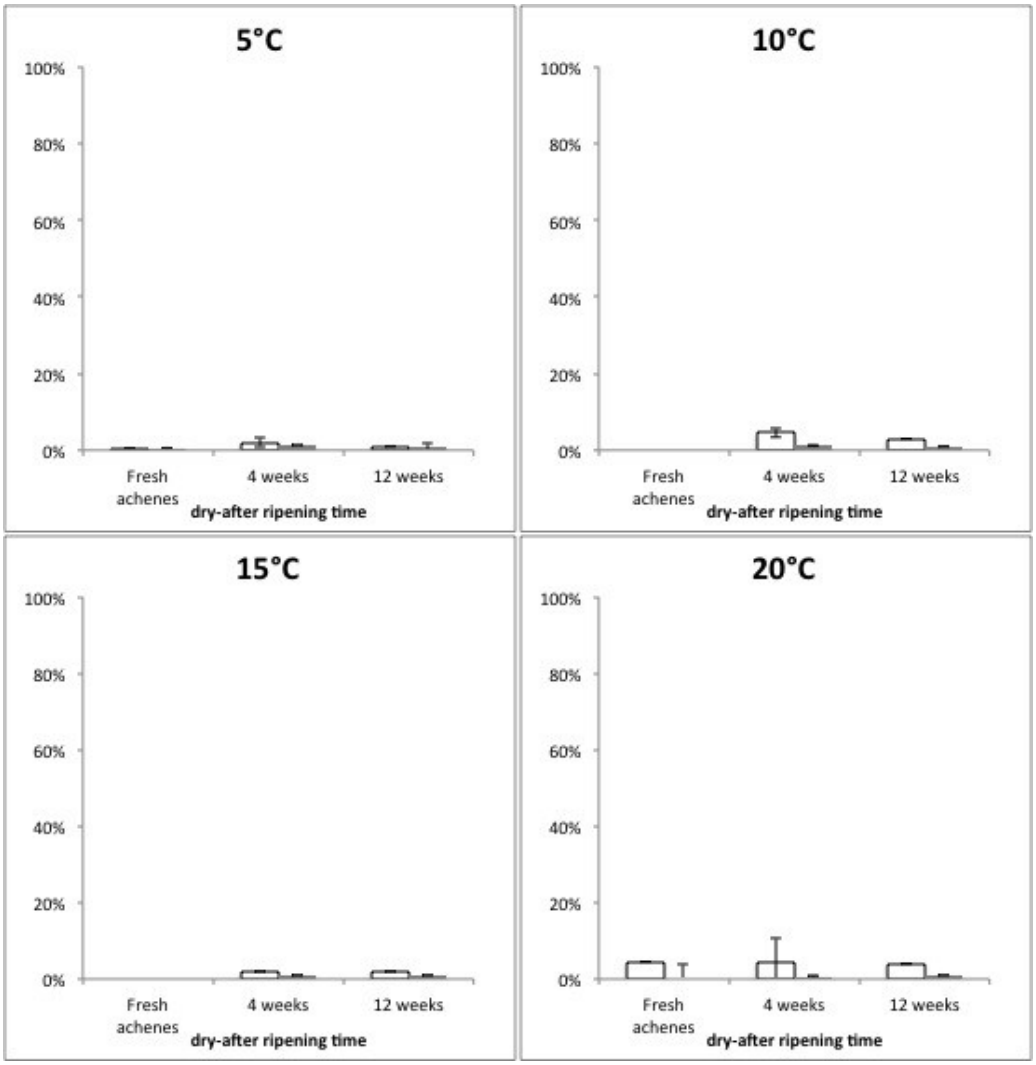

Figure 28 *Chrysanthemum coronarium* var. concolor peripheral achenes dryied after-ripening with alternating temperatures of 35/20° C (night/day). Fruit coat rupture: open histograms; Seed germination: solid histograms.

Wet warm stratification

Even though achenes were washed properly with chlorine, during the wet-warm treatment the presence moulds had a detrimental effect in seed surviving. In fact, dead seed percentage reached significant proportions especially in longer-term treatments and always higher in peripheral than in central achenes. Nevertheless, in *concolor* variety, in both morphotypes, wet warm stratification increased achenes germination together with the increasing of time period treatment in central and in peripheral ones. On the other hand, an increase in exposure of achenes to warm for longer time periods meant a much more mould proliferation and seeds death, hence. When achenes were transferred to the germination temperatures (5, 10, 15, and 20° C) lower temperatures (5 and 10° C) resulted in higher germination frequency than higher temperatures (15 and 20° C) and it is probably associated with mould-spread inhibition at lower temperatures. *Discolor* variety behaved similarly showing the same germination pattern (Figure 31 and Figure 32).

Increasing temperature of wet-warm stratification up to 45° C (Figure 33) produced a much faster fruit coat rupture but it caused seed death. Conversely, a wet-warm imbibition at 40° C provided less achenes rupture and a high dead seeds rate (Figure 34). In Table 3 the fruit coat rupture results from drying after ripening and warm-wet stratification treatments are compared.

Treatment ID	Morphotype	Temperature	Water	FCR	Standard Dev.
Drying after ripening for 4 weeks	Central	35°/20°	-	9,7%	0,041
Drying after ripening for 4 weeks	Peripheral	35°/20°	-	3,3%	0,015
Drying after ripening for 12 weeks	Central	35°/20°	-	8,3%	0,017
Drying after ripening for 12 weeks	Peripheral	35°/20°	-	2,5%	0,013
Wet warm after ripening for 3 weeks	Central	35°/20°	+	21,4%	0,016
Wet warm after ripening for 3 weeks	Peripheral	35°/20°	+	30,7%	0,096
Wet warm after ripening for 5 weeks	Central	35°/20°	+	55,4%	0,052
Wet warm after ripening for 5 weeks	Peripheral	35°/20°	+	61,5%	0,028
Wet warm after ripening for 8 weeks	Central	35°/20°	+	72,4%	0,031
Wet warm after ripening for 8 weeks	Peripheral	35°/20°	+	77,8%	0,032

Table 3 Effect of wet-warm treatment and drying after ripening in fruit coat rupture.

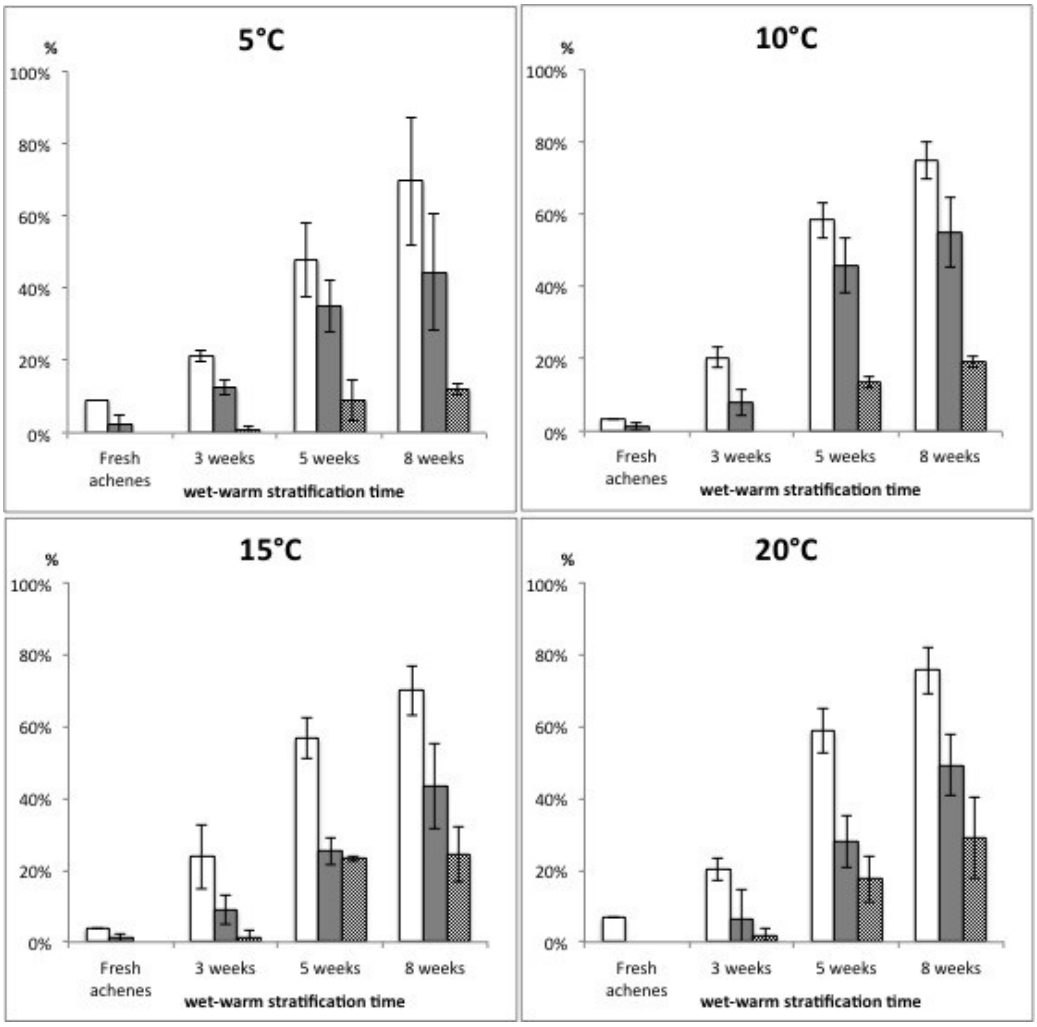

Figure 29 Wet warm stratification after ripening in *C. coronarium* var. *concolor* central achenes. Fruit coat rupture: open histograms; Seed germination: solid histograms; Dead seeds: dotted histograms. In control experiment no seed death was observed.

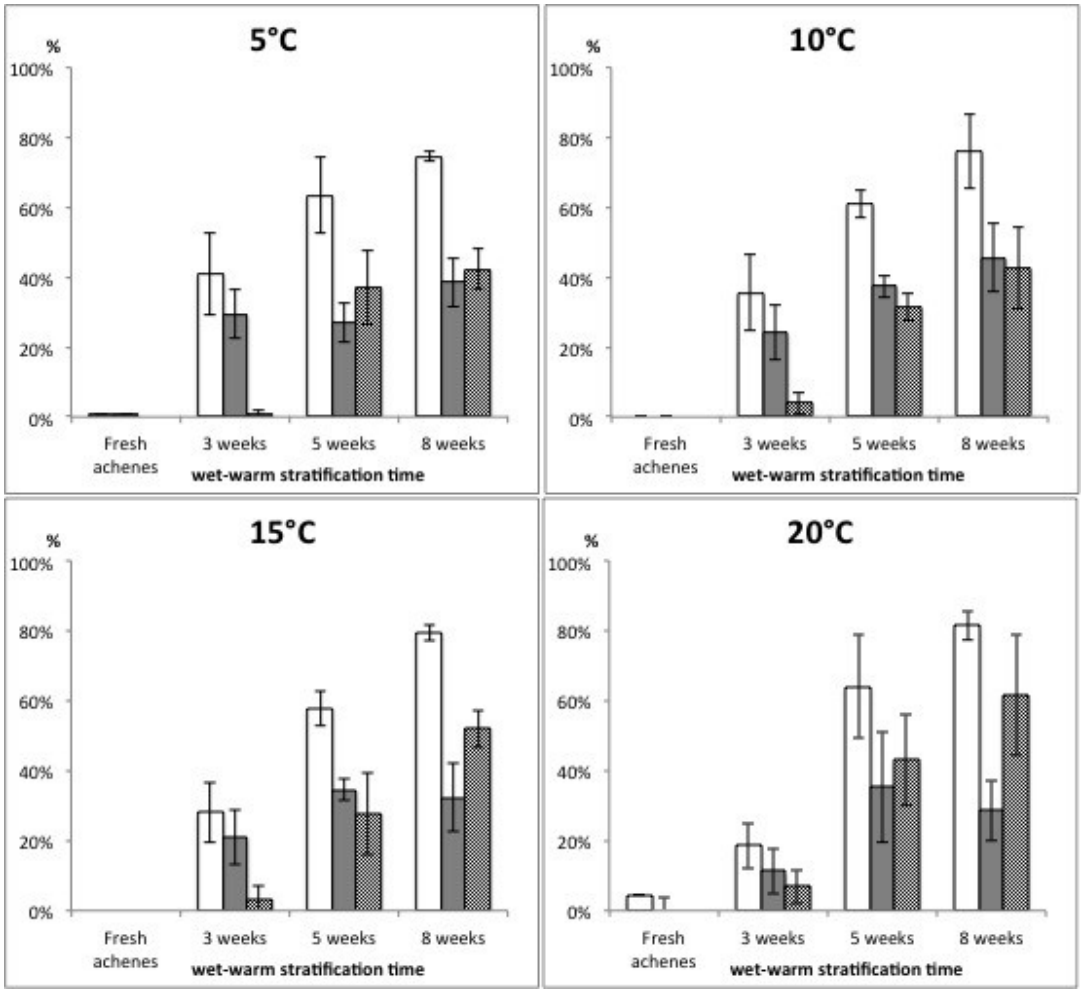

Figure 30. Wet warm stratification after ripening in *C. coronarium* var. *concolor* peripheral achenes. Fruit coat rupture: open histograms; Seed germination: solid histograms; Dead seeds: dotted histograms. In control experiment no seed death was observed.

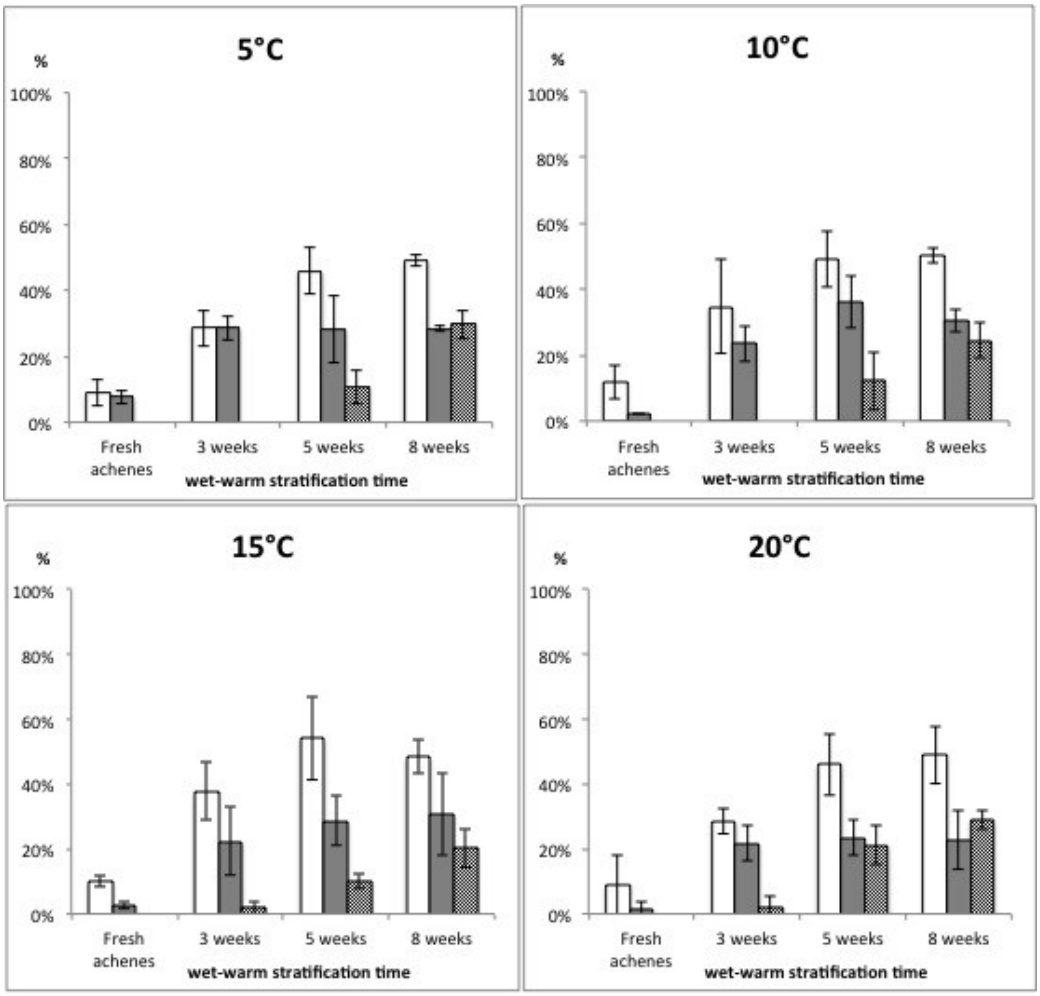

Figure 31 Wet warm stratification after ripening in *C. coronarium* var. *discolor* central achenes. Fruit coat rupture: open histograms; Seed germination: solid histograms; Dead seeds: dotted histograms. In control experiment no seed death was observed.

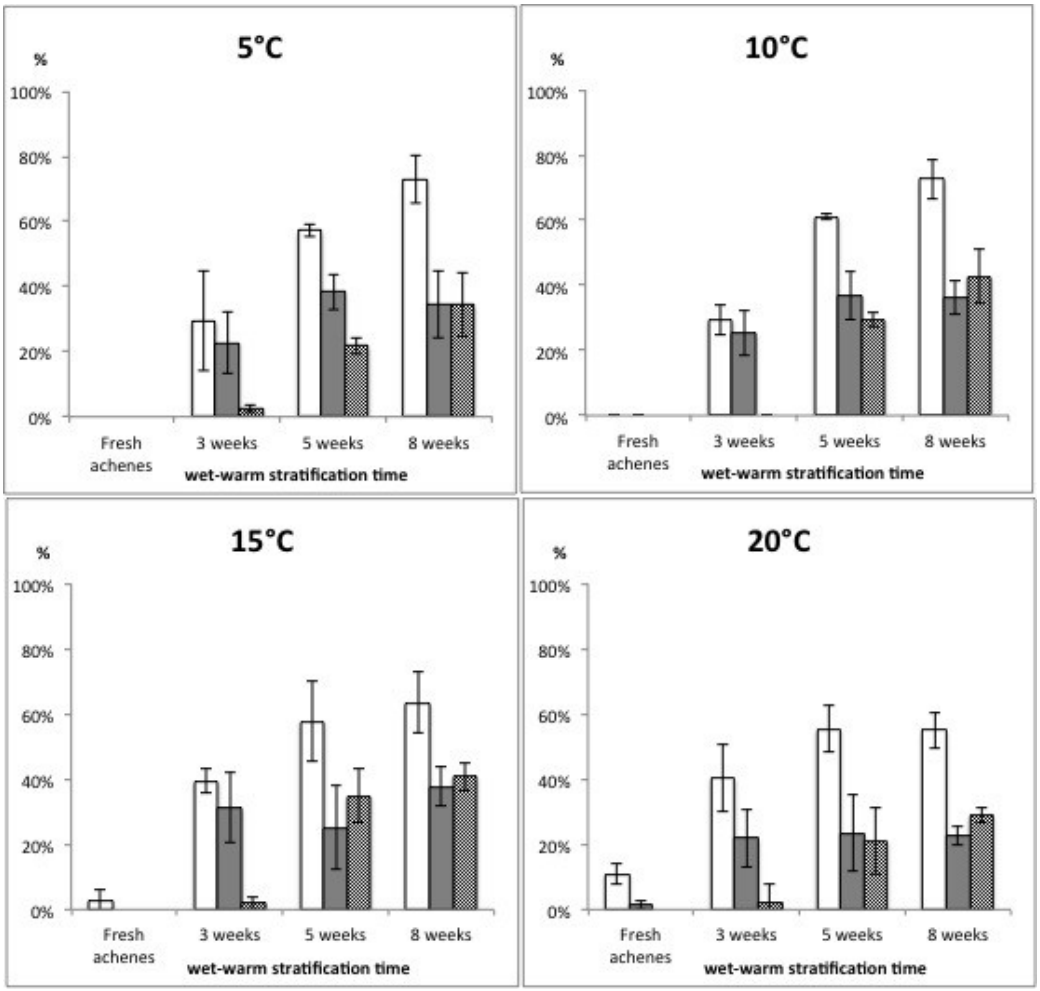

Figure 32 Wet warm stratification after ripening in *C. coronarium* var. *discolor* peripheral achenes. Fruit coat rupture: open histograms; Seed germination: solid histograms; Dead seeds: dotted histograms. In control experiment no seed death was observed.

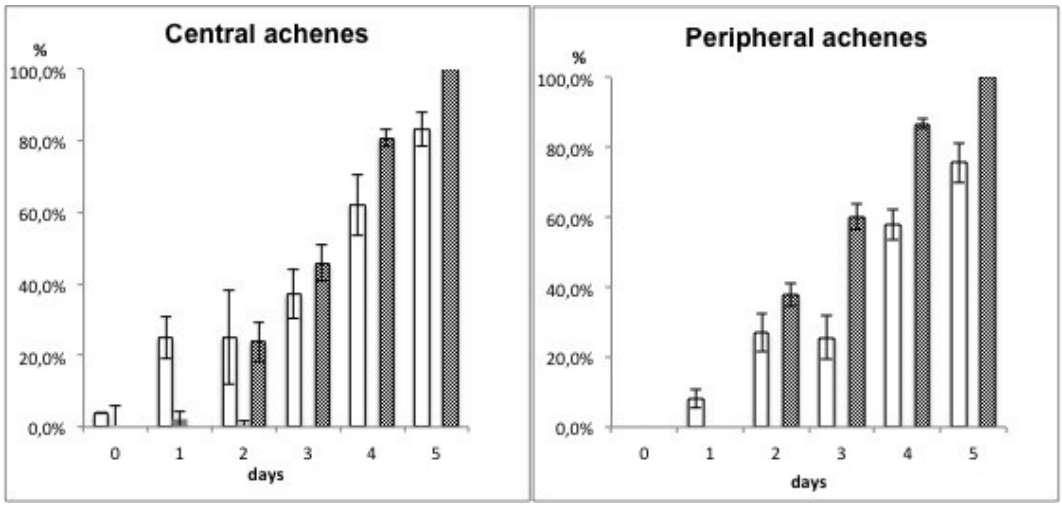

Figure 33 *C. coronarium concolor* wet-warm stratified at 45° C for 1, 2, 3, 4 and 5 days. Fruit coat rupture: open histograms; Seed germination: solid histograms; Dead seeds: dotted histograms.

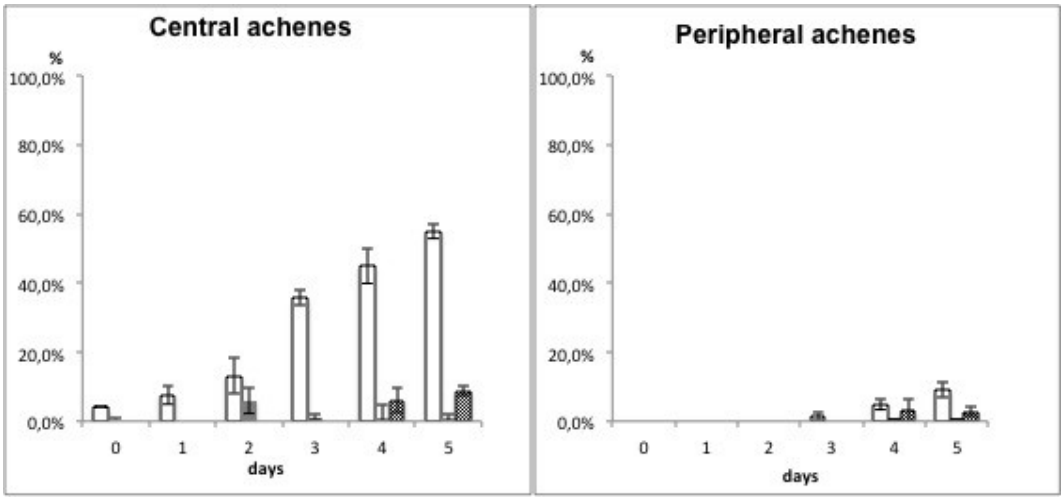

Figure 34 *C. coronarium concolor* wet-warm stratified at 40° C for 1, 2, 3, 4 and 5 days. Fruit coat rupture: open histograms; Seed germination: solid histograms; Dead seeds: dotted histograms.

ABA synthesis

Achenes imbibed at 20° C with different concentrations of fluridone resulted in low germination response (< 5%). The same germination profile was observed when achenes were imbibed with 100uM fluridone after washing for 24h in agitated water.

Genotyping of *Chrysanthemum coronarium concolor* and *discolor* varieties

DNA extraction

An average yield of 3.5 μg of total DNA was obtained from DNA extraction (Figure 35 and Figure 36). Diluted samples resulted in better amplifications (more clear and pronounced bands), but the presence of polyphenol compounds inhibited the *Taq* polymerase activity. Although samples were precipitated and washed again with ethanol the most effective result was obtained by the addition of PVP into PCR mix (Figure 37).

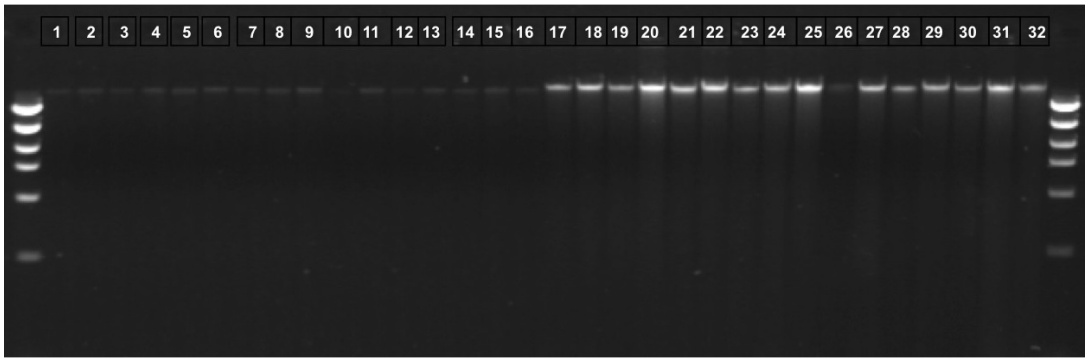

Figure 35 DNA extraction of *Chrysanthemum coronarium* var *concolor*. From 1 to 16 1:10 dilution of extracted samples; from 17 to 32 undiluted samples.

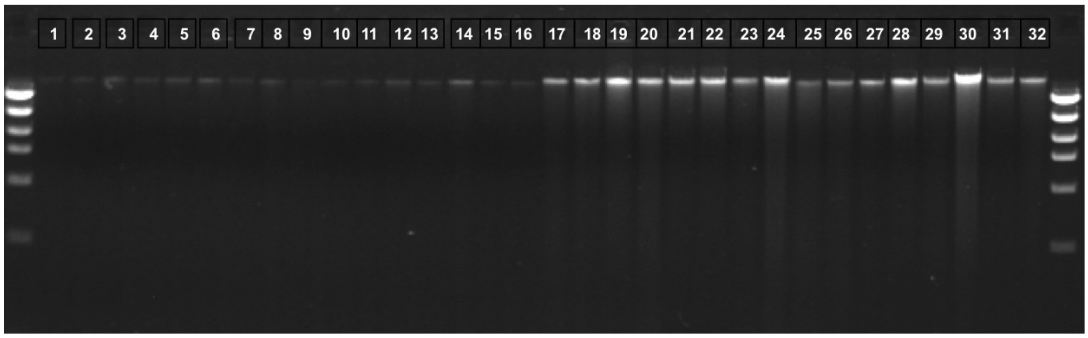

Figure 36 DNA extraction of *Chrysanthemum coronarium* var *discolor*. From 1 to 16 1:10 dilution of extracted samples; from 17 to 32 undiluted samples.

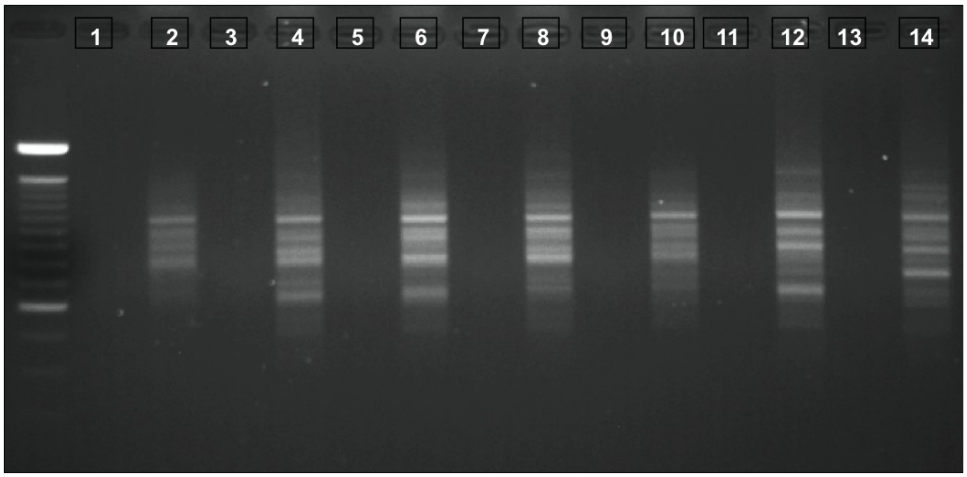

Figure 37 *Chrysanthemum coronarium* var *discolor* amplified with primer Cc_04. 1-2: Cd_10; 3-4: Cd_11; 5-6: Cd_12; 7-8: Cd_13; 9-10: Cd_14; 11-12: Cd_15; 13-14: Cd_16. Odd numbered wells are samples where PVP was not added into PCR mix; Even numbered wells are samples where PVP 2% was added into PCR mix.

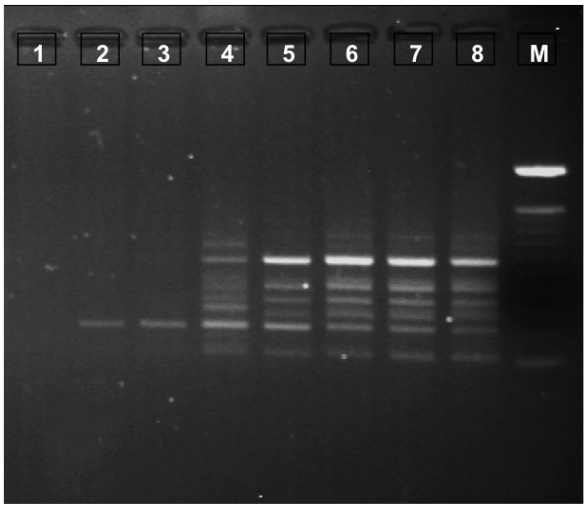

Figure 38 *Chrysanthemum coronarium* var *discolor* amplified with primer Cc_04. Wells 1-4: amplification of Cd_14 sample with, respectively, 0, 1, 1.5, and 2% of PVP in PCR mix; Wells 5-8: amplification of Cd_13 sample with, respectively, 0, 1, 1.5, and 2% of PVP in PCR mix.

Different amounts of PVP in PCR reactions were tested and the addition of 2% PVP produced an increased amount and clearer bands (Figure 38).

ISSR analyses

The final ISSR matrix included 32 individuals and 40 loci, 27 of which were diallelic. We performed a principal co-ordinate analysis (PCoA) of this binary matrix, based on the Euclidean distance, to represent the genetic relationships among populations and individuals. Because of their capability to represent genetic differentiation, ordination axes obtained from neutral loci matrix can be used to assess correlations between genetic composition and other variables (Lee 2011).

Sequencing of *rbcL* gene

The large subunit of ribulose-bisphosphate carboxylase (rbcL) gene of 1206 nt was amplified for *concolor* and *discolor* variety of *Chrysanthemum coronarium* (Figure 39). PCR products were directly sequenced using amplification primers.

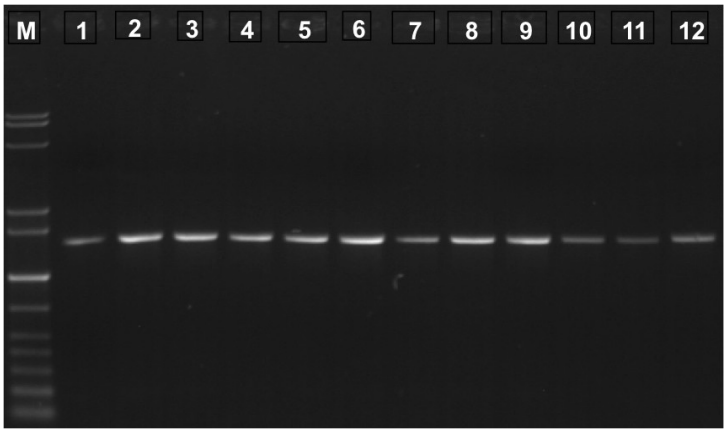

Figure 39 Amplification of *rbc-L* gene in six samples per each variety. 1-6: *C. coronarium concolor* variety; 7-12: *C. coronarium discolor* variety.

Physiology of germination in *Leucanthemum vulgare*

Germination behaviour

Leucanthemum vulgare seeds imbibed in water did not showed any dormancy-associated behaviour and germinated in a great amount. In particular, they showed the highest germination percentage when imbibed at 15 - 25° C temperature range (Figure 40) with the probable optimum around 20° C.

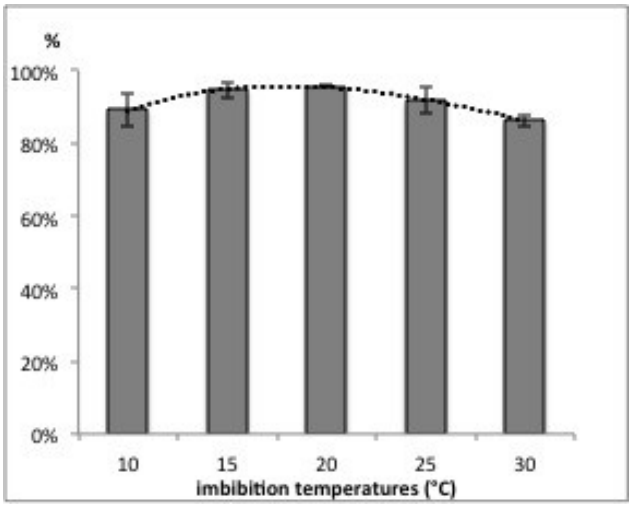

Figure 40 Germination response of *Leucanthemum vulgare* seeds at different imbibition temperatures

Seed osmo-priming

Interestingly, sowing seeds at 10° C after a one-week priming treatment (short-priming treatment) the germination response decreased dramatically (Figure 41). Seeds sowed at higher temperatures were not inhibited by priming, while at 10° C, germination percentage showed a remarkable decrease (-83,2%). Priming seeds for nine weeks (long priming treatment) at 5° C, produced a reversion in germination response and treated seeds behaved as control experiment. Ten-degrees primed seeds, on the other hand, retained priming inhibition in the long-priming treatment as well. Furthermore, nine-weeks priming produced a lower germinability at 30° C, where a significant (26% for 5° C primed seeds and 34% for 10° C primed seeds) of germination drop was observed.

Light during short and long priming treatments produced an increase just in seeds primed at 10° C and imbibed at 10° C where it doubled the germination response in comparison with treatment in darkness (Figure 42).

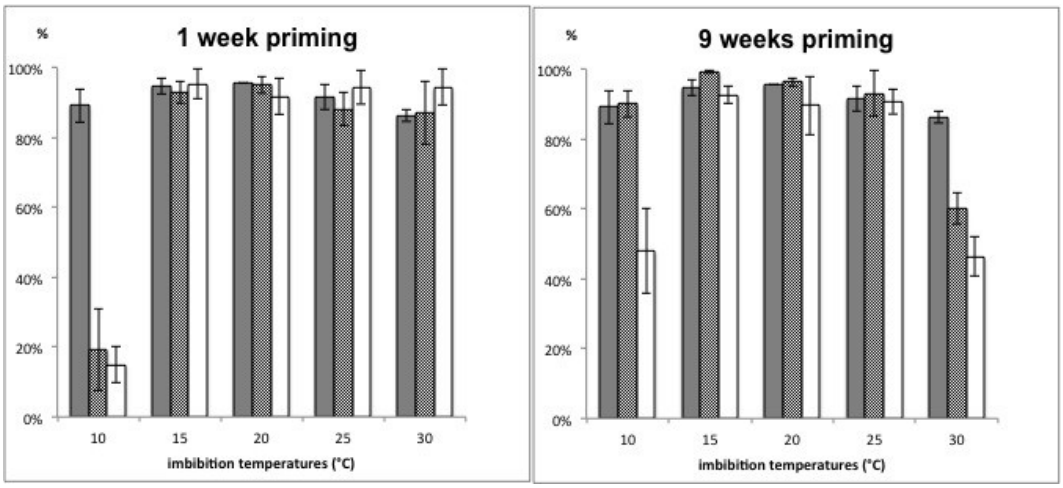

Figure 41 Comparison of the effect of short and long priming treatment on germination behaviour. Solid histograms: imbibition in water (no priming); Dotted histograms: 1 week primed seeds at 5° C; open histograms: 1 week primed seeds at 10° C. In this experiment the priming treatment was carried out in darkness.

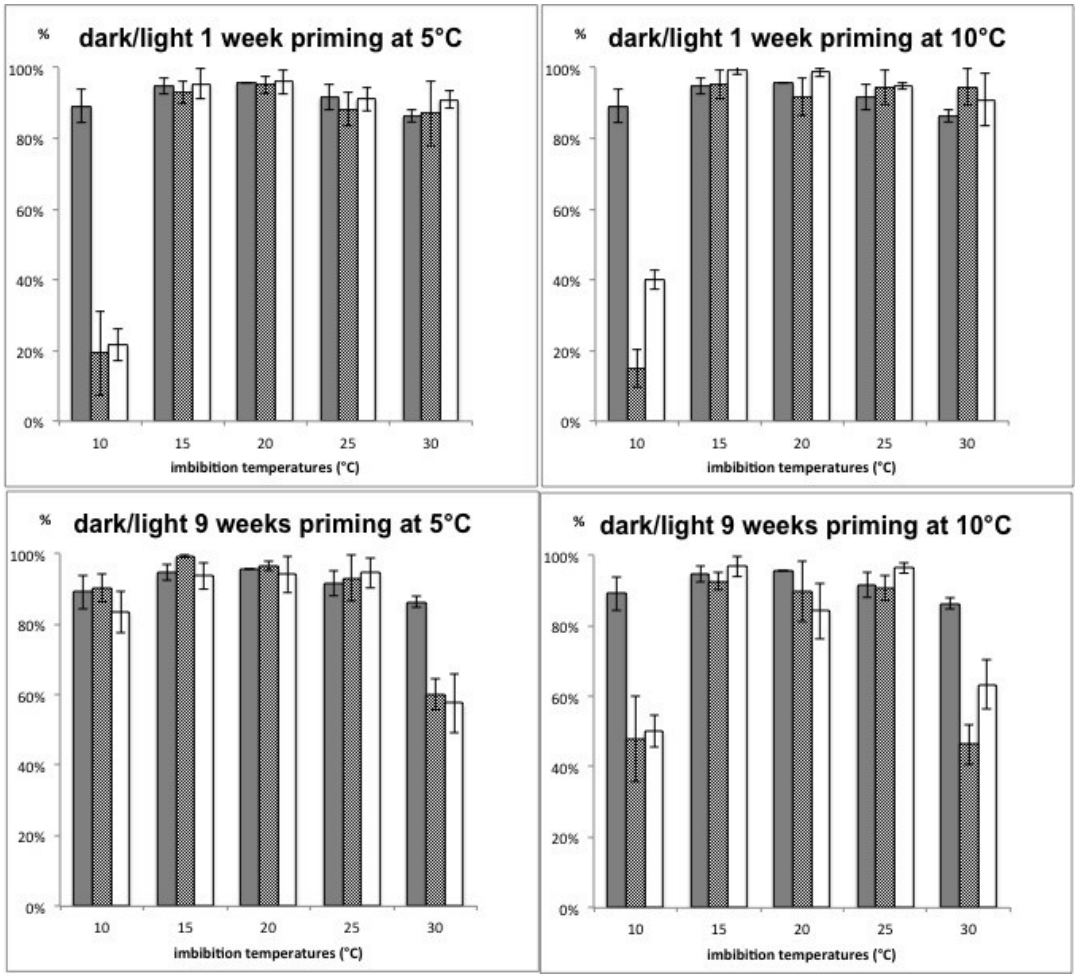

Figure 42 Short and long seed priming with light or darkness. Dotted histograms are dark primed whereas open histograms are primed in light.

Hydro-priming

Sowing seeds at 10° C after a one-week incubation in darkness at 5° C caused a dramatic drop in germination irrespective of osmotic potential of the medium. On the other hand at higher germination temperatures hydro-priming does not produced any remarkable effect (Figure 43).

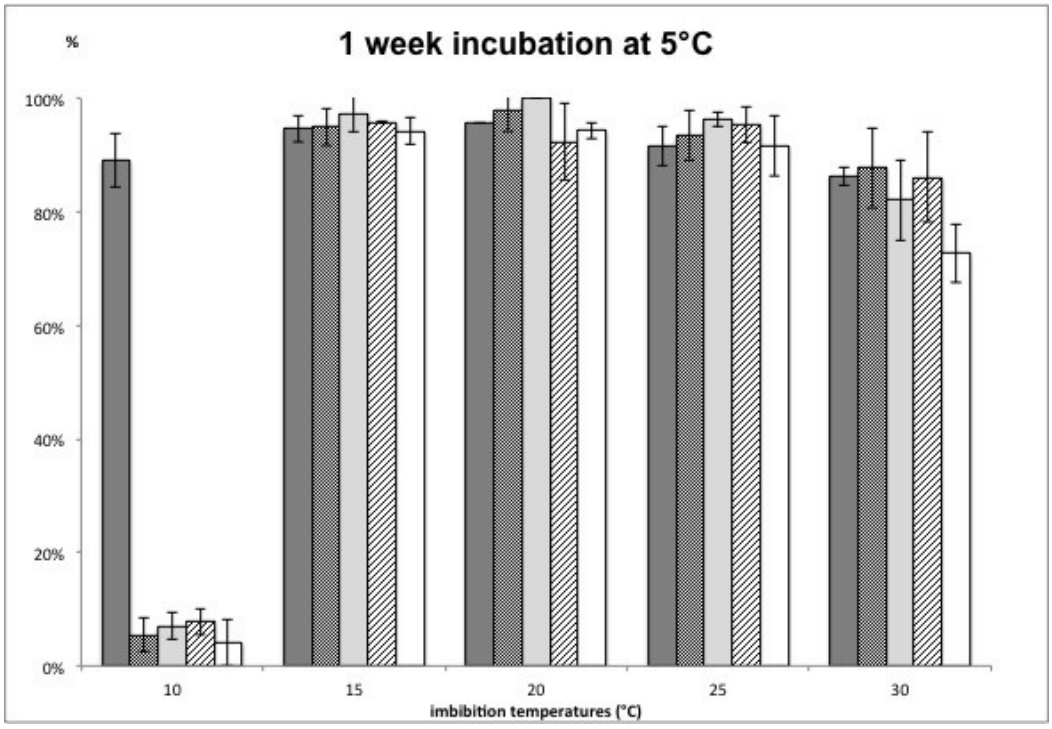

Figure 43 Seed germination of seeds incubated for 1 week at 5° C at different conditions: Solid histograms non-treated seeds; Dotted histograms: seeds imbibed for 1 week in water in dark; Light gray histograms: seeds imbibed in darkness for 1 week with an osmotic potential of ψ=-0.02; Striped histograms: seeds imbibed in darkness for 1 week with an osmotic potential of ψ=-0.072; Open histograms: seeds imbibed in darkness for 1 week with an osmotic potential of ψ=-0.292.

ABA synthesis

Imbibition with fluridone did not produced any significant seed germination increase. Although during osmo-priming seeds were exposed to a low osmotic potential medium and it caused a drop in germination at 10° C, when seeds where sowed with similar osmotic potential conditions (-0.069 respect to -0.072 MPa) seed germination was just partially lowered (from 89 to 60%) (Figure 44).

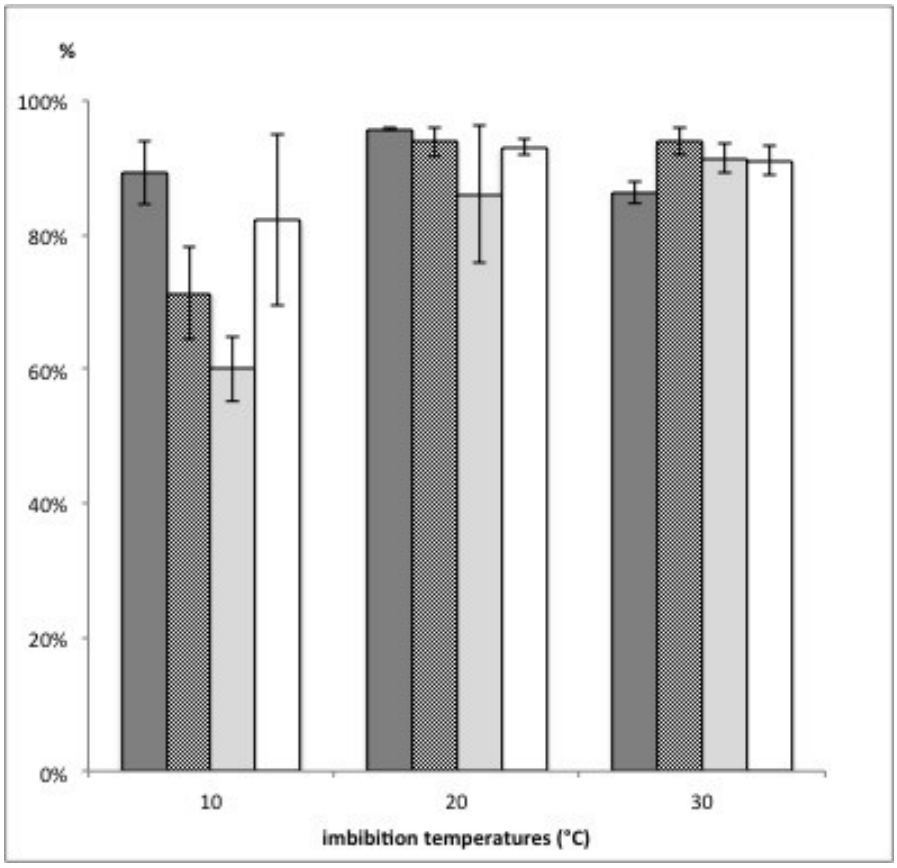

Figure 44 Comparison of germination response among control experiment (solid histograms, imbibition in water), fluridone presence (dotted histograms, imbibition in fluridone 50uM), low osmotic potential medium (light grey histograms, imbibition with a ψ respecively of -0.069, -0.065, and -0.060 MPa) and fluridone together with a low osmotic potential medium (open histograms, imbibition with fluridone 50uM with a ψ respecively of -0.069, -0.065, and -0.060 MPa).

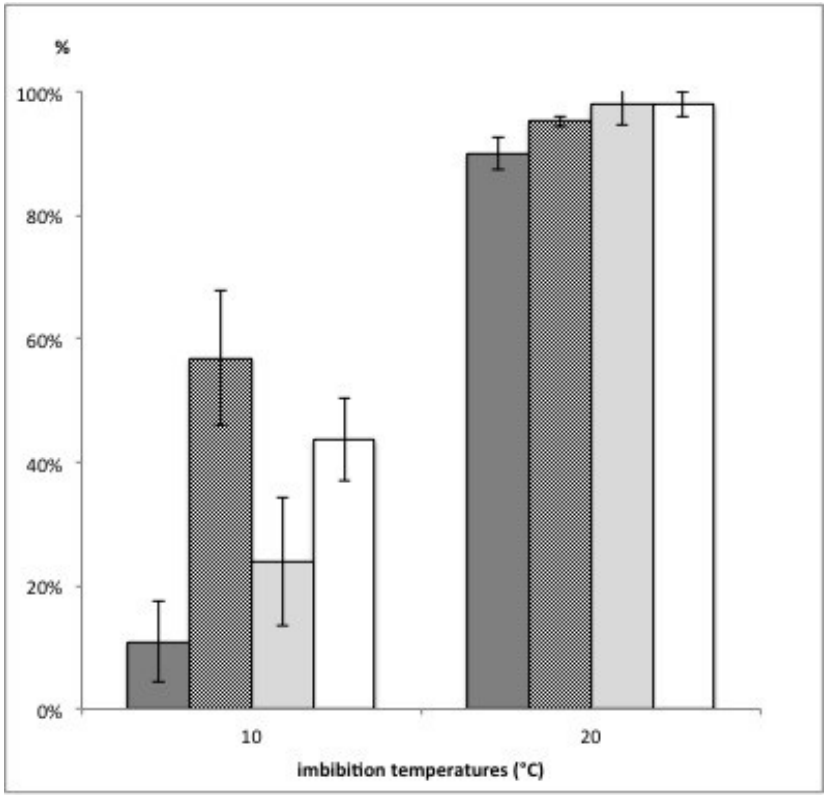

Figure 45 Comparison of germination response among one-week primed seeds at 5° C (solid histograms), one-week primed seeds at 5° C afterwards imbibition with fluridone 50uM (dotted histograms), one-week primed seeds at 5° C with fluridone 50uM afterwards imbibed in a low osmotic potential medium -0.069 MPa (light grey histograms), one-week primed seeds at 5° C with fluridone 50uM afterwards imbibed with fluridone 50uM(open histograms,).

Further experiments with fluridone and giberellic acid in primed seeds gave significant responses only when one-week primed seeds were imbibed with fluridone 50µM at 10° C (+42%) and with giberellic acid 1µM at 10° C (+22%) (see Figure 45 and Figure 46).

Analysis of correlation between giberellic acid/fluridone concentration and germination percentage showed a positive giberellic acid dose effect ($R^2 = 0,76$) with 5 weeks primed seeds and no correlation for fluridone (Figure 47).

Test	Conditions before germ.	Germination cond.	Germination %	Δ %
Control	-	10° in water	89,2%	-
1w dark	1 week in dark at 10°	10° in water	6,0%	-83,2%
1w PEG at 5°	1 week in PEG at 5°	10° in water	19,0%	-70,2%
1w PEG at 10°	1 week in PEG at 10°	10° in water	15,0%	-74,2%
5w PEG at 10°	5 week in PEG at 10°	10° in water	31,3%	-57,9%
9w PEG at 5°	9 week in PEG at 5°	10° in water	90,0%	+ 0,8%
9w PEG at 10°	9 week in PEG at 10°	10° in water	48,0%	-41,2%

Table 4 Comparison of germination percentage for seeds with different treatments before imbibition

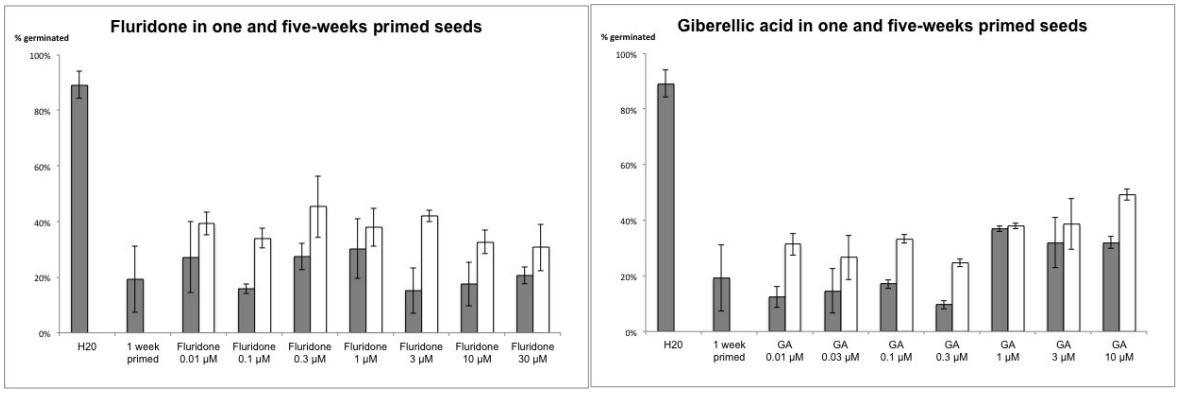

Figure 46 Comparison of different concentrations of fluridone and giberellic acid in one-week primed seeds (solid histograms) and five-weeks primed seeds (open histograms). Final imbibition was carried out at 10° C.

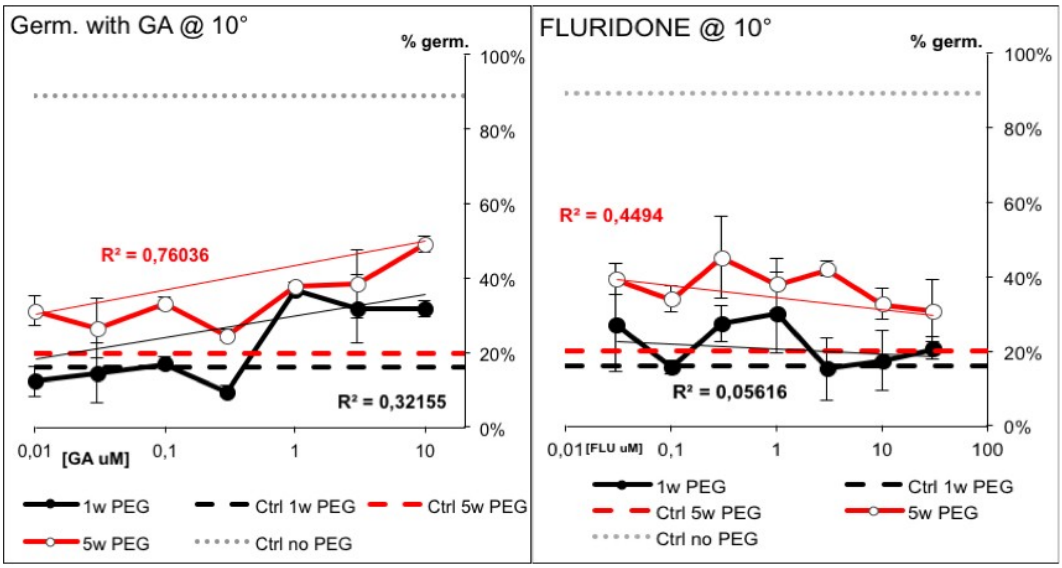

Figure 47 Correlation between giberellic acid/fluridone concentration and germination percentage

Isolations of dormancy genetic markers in *Leucanthemum vulgare* and *Chrysanthemum coronarium*

Genes selection

Five most stable genes were selected as reference genes and their expression level was analysed in a time course graph (Figure 48). Four "Ubiquitin carrier protein" (UBC) genes 8, 12, 21, and 29 together with a "Phospho-glycerate kinase" family protein (PGK) gene, and the 18S ribosomal RNA were selected. From these sequences, Asterids homologous sequence was obtained.

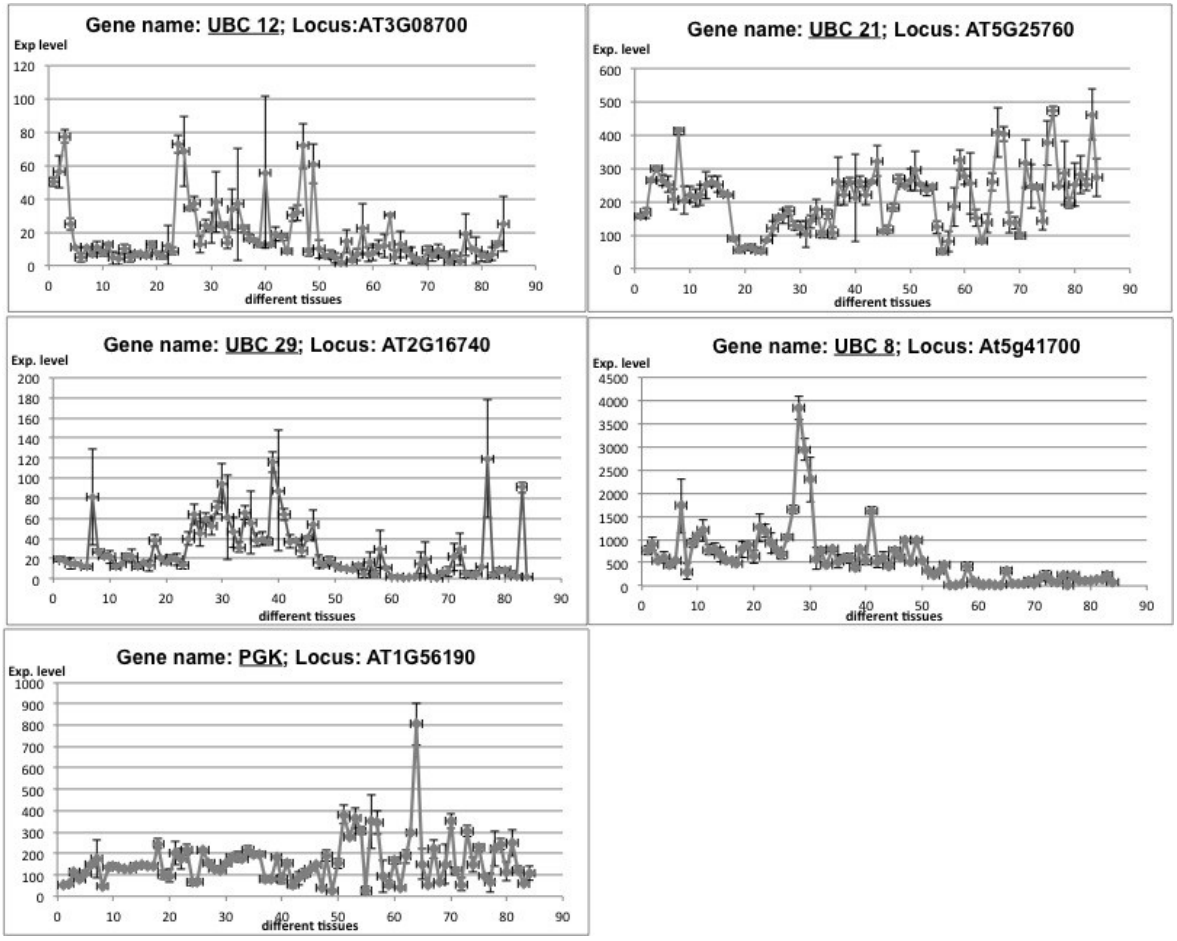

Figure 48. Expression level analysis of reference genes in different tissue and developmental stage in *Arabidopsis thaliana* (rough data from http://www.arabidopsis.org/ web portal).

For dormancy-associated genes, *DOG1*, *FLC*, and *HUB2* Asterids homologous sequences were obtained.

Starting from homologous sequences alignments 62 new cloning were designed and 42 (Table 5) of which were used for PCR amplifications.

Oligo ID	3'-5' Primer sequence	Description	Use in this study
UBC_9	GATTTTGAAGGAATTGAAGG	ubiquitin-conjugating enzyme	reference gene
	TACTTCTGTGTCCAGCTACG		
UBC_10	TGGCTTCGAAAAGGATTTTG	ubiquitin-conjugating enzyme	reference gene
	TGGCGTACTTCTGTGTCCAG		
UBC_8	ATGGCTTCAAAGCGGATCTT	ubiquitin-conjugating enzyme	reference gene
	CAGCTGCGTGCAGTAGTTTC		
UBC_28	GAAAGAGCTCAAGGATCTTC	ubiquitin-conjugating enzyme	reference gene
	CCATGGCATACTTTTGG		
	CAAAGCGGATCTTGAAA		
	GCATACTTTTGGGTCCAG		
UBC_21	AGGCATCAAGAGCAAGACT	ubiquitin-conjugating enzyme	reference gene
	CTGCAAGCTTGGTATACATC		
	TGCAGGCATCAAGAGC		
	AGCCATAGACTGAAATCCTC		
PGK	GAAGAACGATCCAGGCTTTG	phosphoglycerate kinase	reference gene
	CACCCATCTGGGATAGCAGT		
	CTTCCTGATGGTGGTGTT		
	ATCTGGGATAGCAGTGGA		
18s RNA	CTCAAAGATTAAGCCATGC	ribosomal component	reference gene
	TACGAGCTTTTTAACTGCAA		
	GAGCTAATACGTGCAACAAA		
	CCTTACTATGTCTGGACCTG		
DOG1_f1	CTAAGGAAAATGGTGAACAA	Delay of Germination	Dormancy-associated gene
	TCTCTTTGTTGCTGCTAAGT		
	ACGACGAATTGAATGAGTT		
	ATATGCCCAATATCTCCTTT		
	TTGAATGAGTTACTAAG		
	TCTTCTAGTAAATTTGACAT		
FLC_f1	ATCGATATGGGAAACA	Flowering Locus C	Dormancy-associated gene
	AWCGATATSGSAAMCA		
	GCTCTTCTCGTCGTCTC		
	GTCTTCTTGGYTCTRRT		
Hub2_f1	CTCCAGCATCAAAATCAGA	Histone H2B Monoubiquitination	Dormancy-asociated gene
	TTCAGCTGAGCTTCCATCAT		
	AAAATGCAGAAAGATGTGGCA		
	ATGATGGAAGCTCAGCTGAA		
	ATTACAACCTCTTTTGGCC		
	ATGATGGAARCWCAGYTRAA		
	ATTACMACCTCYTTKGGYC		
EXP	ACGACCCTAAATGGTGCTTG	Expansin	Dormancy-associated gene
	TTGTGATGGTTCTTCCGTCA		

Table 5 Cloning primers list of dormancy-associated genes and reference genes

DNA extraction

Four different replicas were obtained from DNA extraction and a 1:1000 dilution was used as template for gDNA quality control with Expansin primers designed for *Arabidopsis thaliana* in PCR (Figure 49).

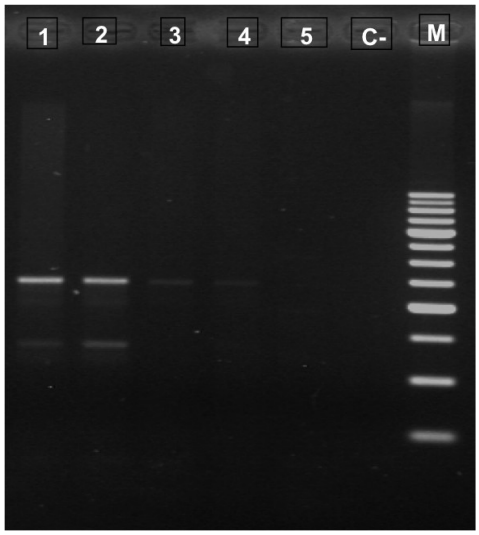

Figure 49 PCR amplification of extracted gDNA. 1 = *L. vulgare* replica 1 (with testa tissue); 2 = *L. vulgare* replica 2 (with testa tissue); 3 = *L. vulgare* 1:1000 diluition template 1 (without testa tissue); 4 = *L. vulgare* 1:1000 diluition template 2 (without testa tissue); 5 = *Succisa pratensis* DNA extraction. C- = PCR negative control; M = marker.

RNA extraction

Extracted RNAs from various treated tissues were quantified and analysed through electrophoresis runs (Figure 50 and Figure 51).

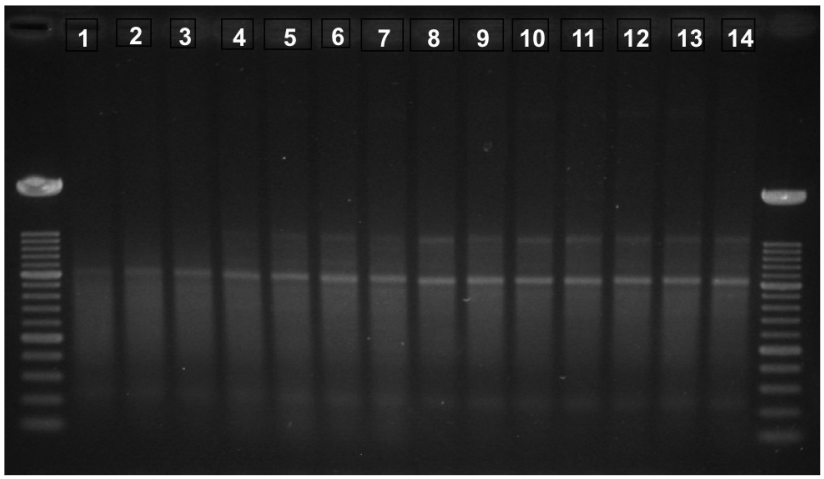

Figure 50. Wells 1-7 = five weeks After drying replicas; 8-10 = 24 hours after imbibition at 30° C replicas; 11-13 = 24 hours after imbibition at 20° C replicas; 14 = 24 hours after imbibition at 10° C replica.

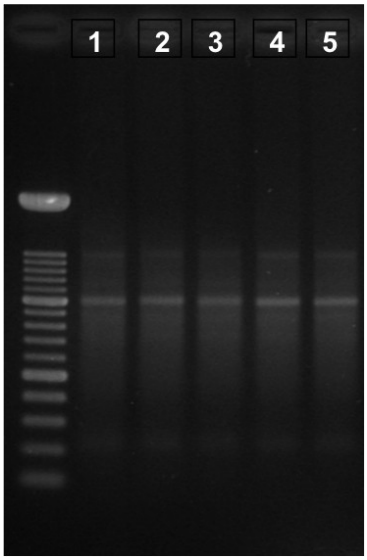

Figure 51. Wells: 1-2 = 24 hours after imbibition at 10° C replicas; 3-5 = 24 hours after imbibition at 20° C replicas.

From the obtained RNAs samples cDNA first synthesis was carried out and checked in a control PCR using *A. thaliana* Expansin primers (Figure 52).

Figure 52. Expansin amplification with cDNA as template

PCR products cloning with TA-Vector

From the gDNA and cDNA of *L. vulgare* and with *C. coronarium* gDNA, reference and dormancy associated genes were amplified. Amplified samples were selected basing on the band analysis (especially taking into consideration the size of the predominant band size compared to the supposed sequence length). Twenty-one PCR products samples from

Chrysanthemum coronarium gDNA amplifications were chosen for plasmid cloning (Figure 53).

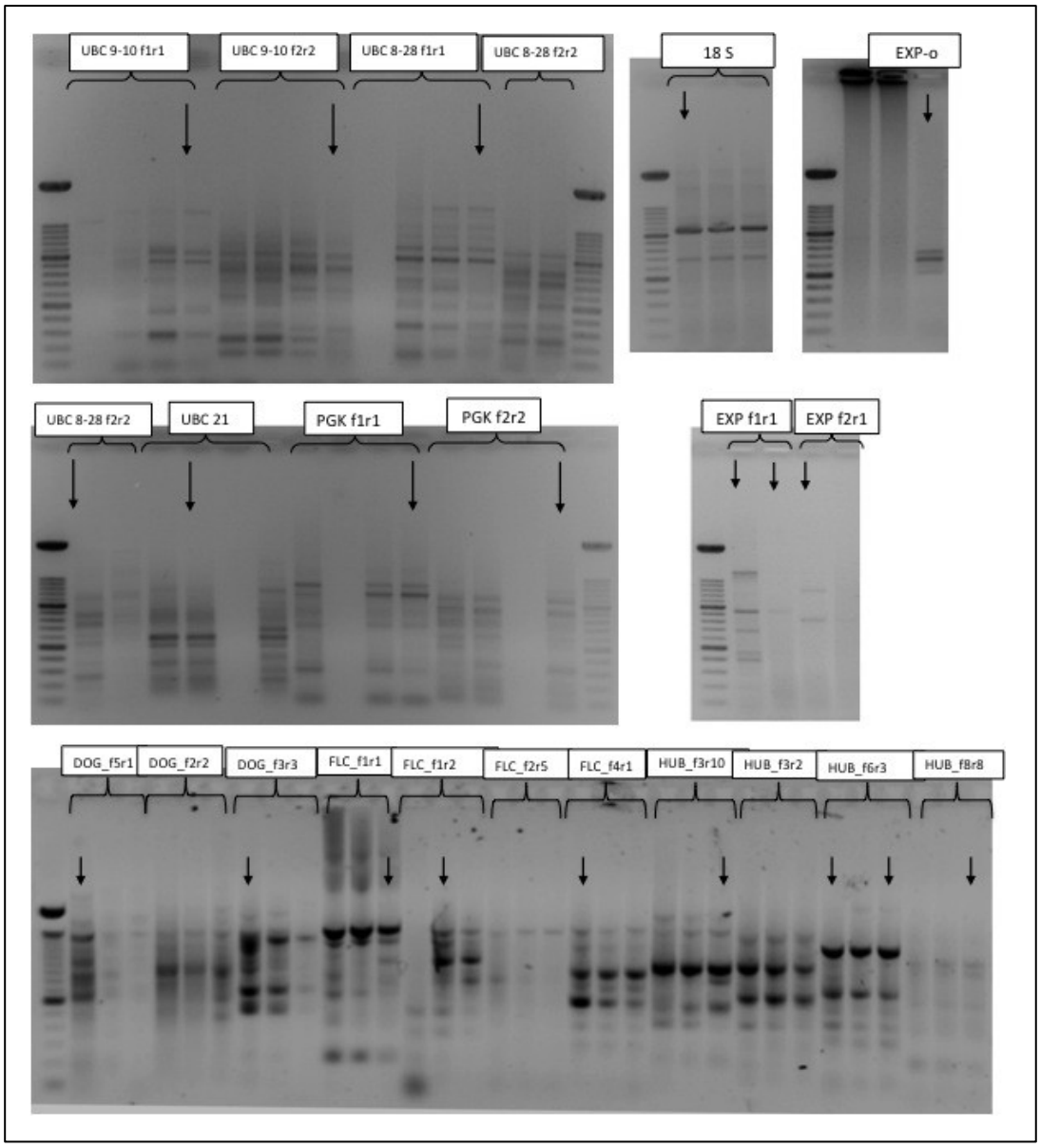

Figure 53 Amplification of reference and dormancy-associated genes using gDNA of *Chrysanthemum coronarium* as template. Arrows indicate the PCR product selected for plasmid cloning.

Likewise, from the amplifications with *Leucanthemum vulgare* twenty-four PCR products samples using gDNA as template (Figure 54), and eight PCR products using cDNA as template (Figure 55) were used for plasmid cloning.

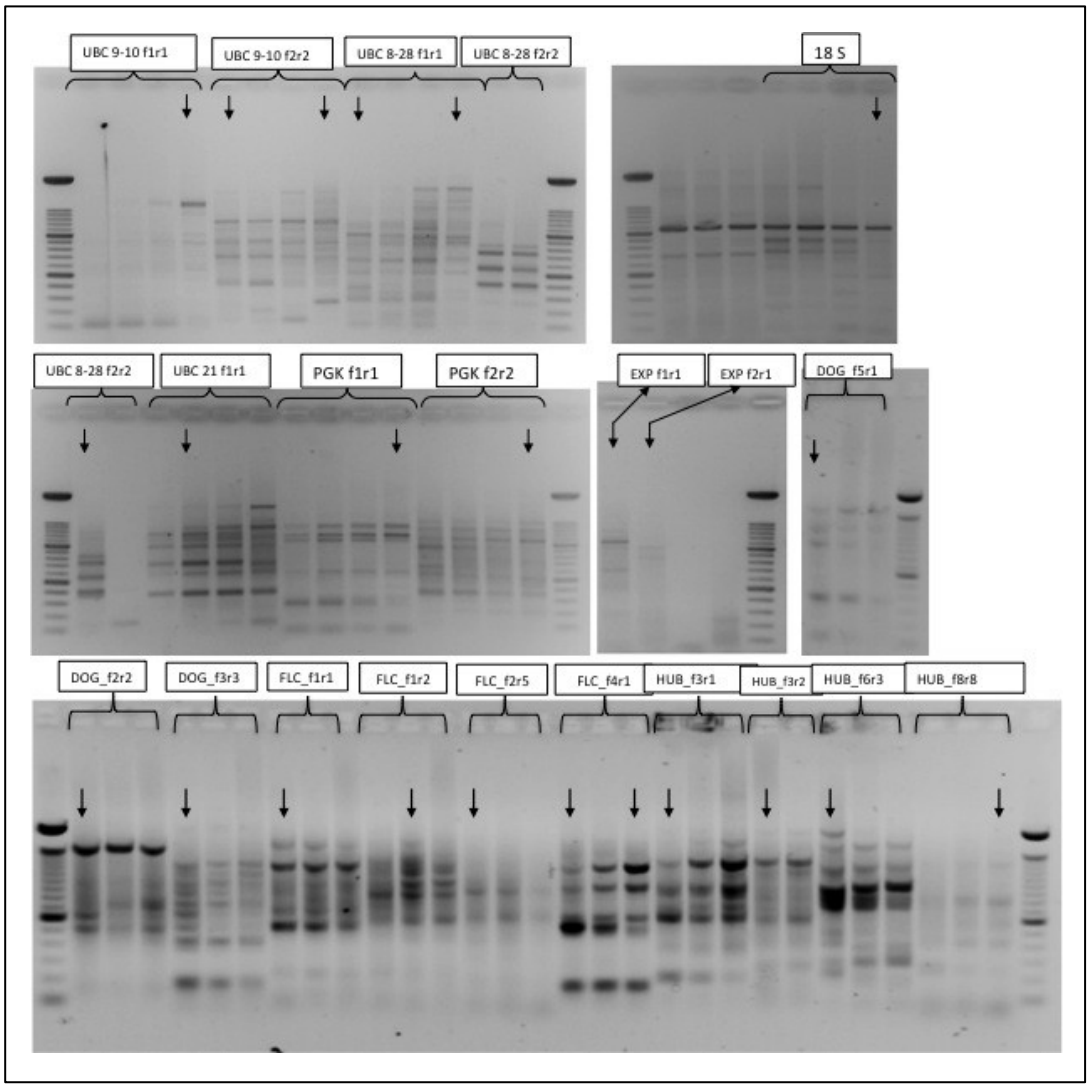

Figure 54 Amplification of reference and dormancy-associated genes using gDNA of *Leucanthemum vulgare* as template. Arrows indicate the PCR product selected for plasmid cloning.

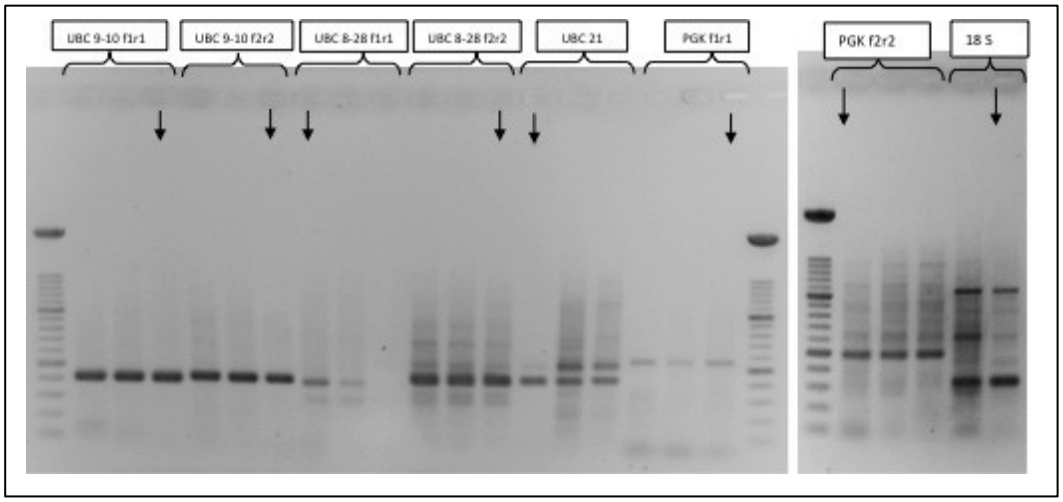

Figure 55 Amplification of reference and dormancy-associated genes using cDNA of *Leucanthemum vulgare* as template. Arrows indicate the PCR product selected for plasmid cloning.

From the PCR colony screening (see Figure 56, Figure 57 and Figure 58 as examples of the colony PCRs carried out), 119 clones were identified for DNA sequencing and 84 of which gave suitable sequence reads.

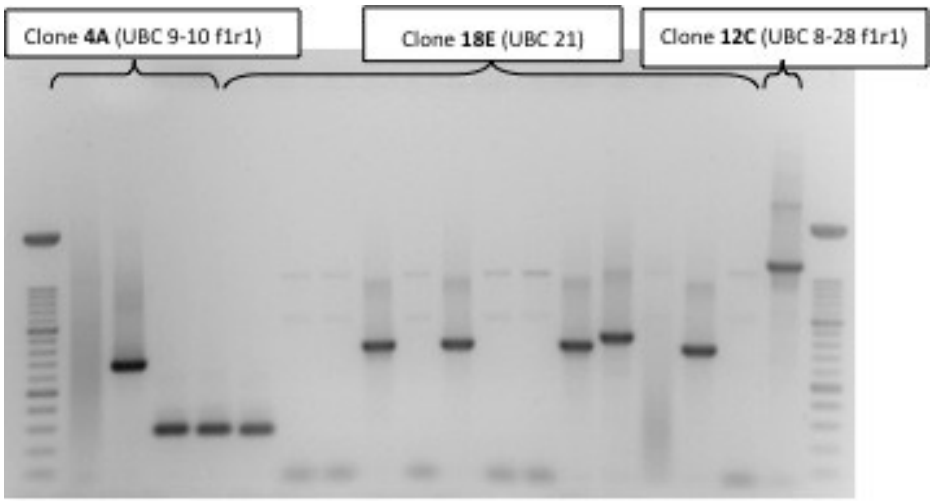

Figure 56 Some of the colony PCRs carried out starting from *Chrysanthemum coronarium* gDNA as template. Colonies with longer inserts were used for DNA sequencing.

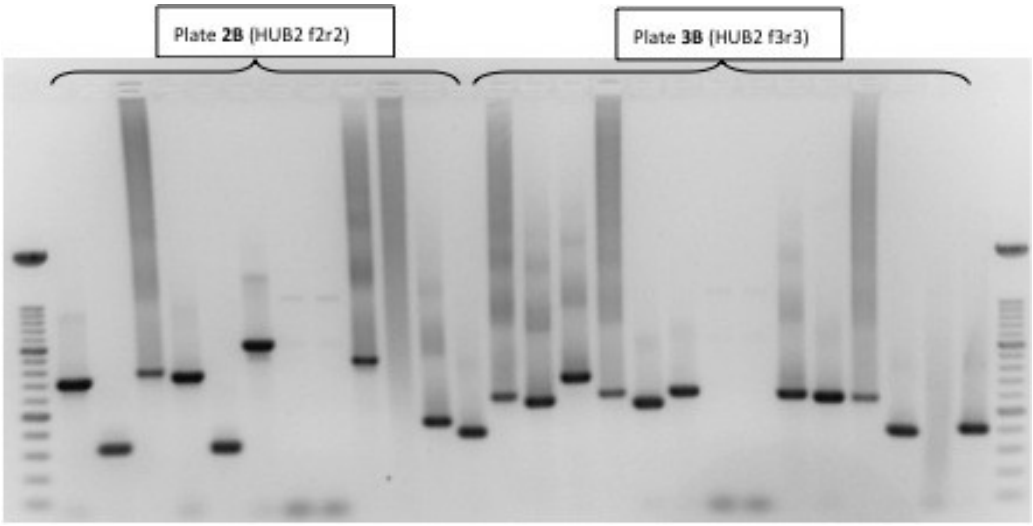

Figure 57 Some of the colony PCRs carried out starting from *Leucanthemum vulgare* gDNA as template. Colonies with longer inserts were used for DNA sequencing.

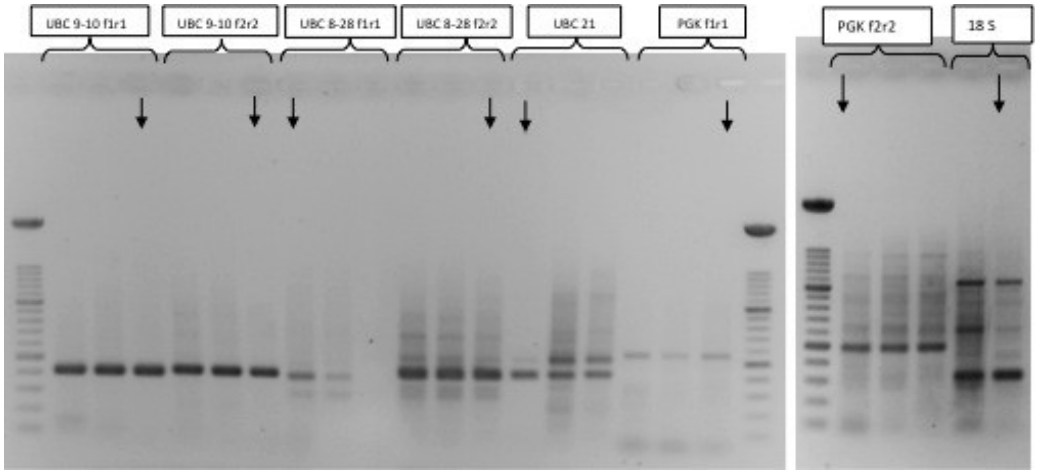

Figure 58 Some of the colony PCRs carried out starting from *Leucanthemum vulgare* cDNA as template. Colonies with longer inserts were used for DNA sequencing.

Sequences analyses

Phosphoglycerate kinase (PGK) gene

Using *C. coronarium* chromosomal DNA as template significant results in BLAST search were obtained. In particular "PT_28-13" sequence provided a specific hit with *PGK* gene. In the alignment with homologous sequences *C. coronarium* clearly clustered with *Helianthus* (Figure 59).

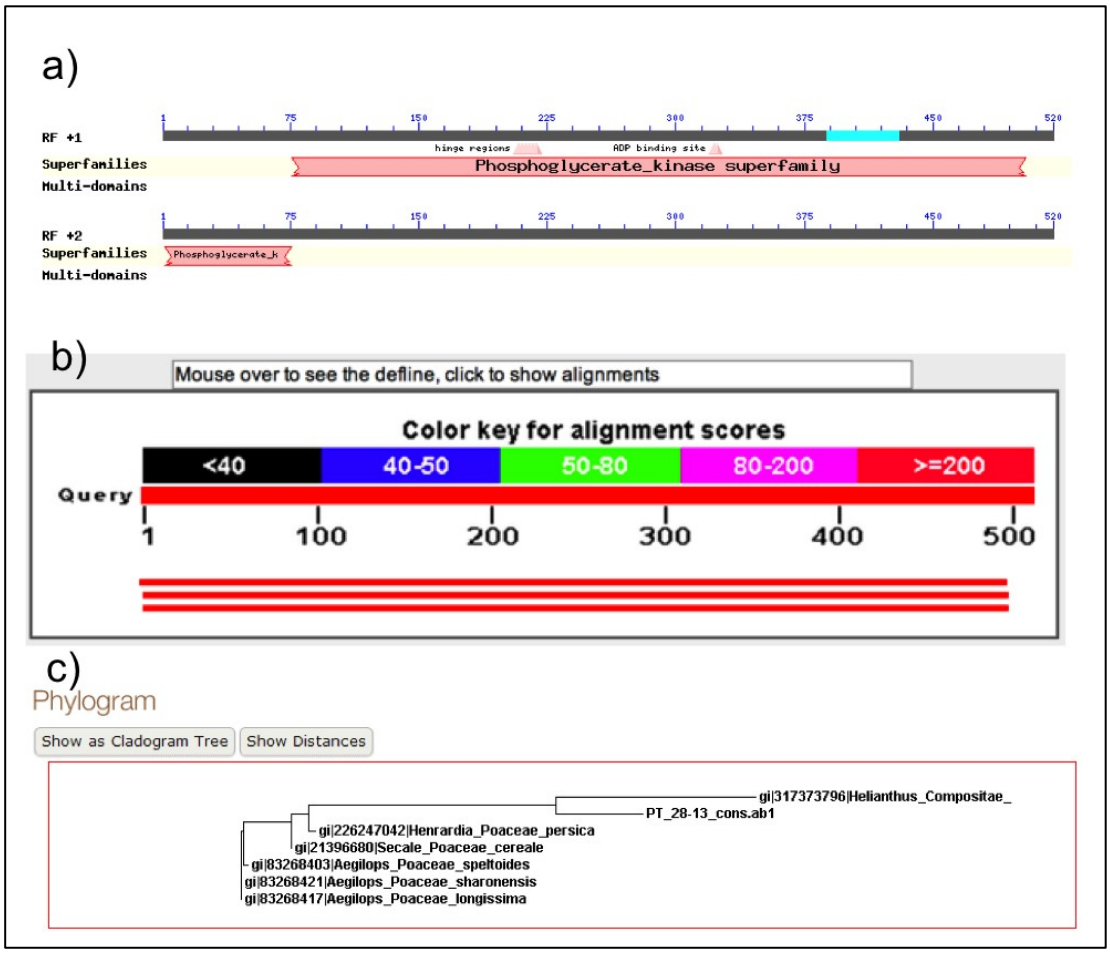

Figure 59 a) Protein domain, BLASTx search; b) Nucleotide BLASTn search; c) Phylogram obtained from sequences alignment.

18s gene:

Both species provided similar results with a high hit score specific for *18S* gene. Alignment with *Helianthus* 18S rDNA sequence showed that sequences are located in the initial part of the gene (Figure 60).

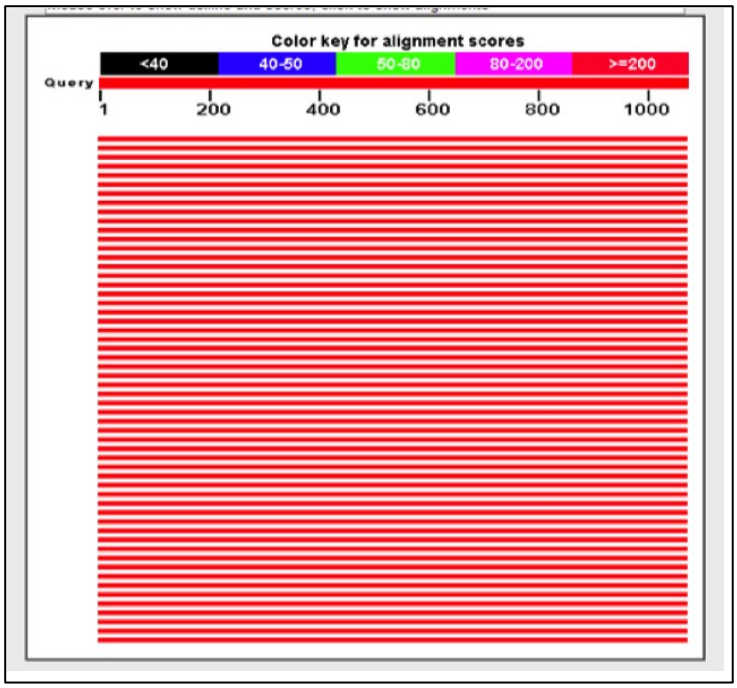

Figure 60 Nucleotide BLAST search of 18S obtained sequences. First hit is *Helianthus anuus*.

UBC 21 gene

For this gene only *L. vulgare* cDNA template provided significant results. Among the obtained sequences, "PT_79_1_T7.ab1" appears to be less *UBC* gene specific than "PT_79_4_T7.ab1" and "PT_79_18_T7.ab1". The reason is that for "PT_79_1_T7.ab1" in position 1005 of original sequence there is a deletion of a single base, which gives a different open reading frame (ORF). PT_79_18 and PT_79_4 sequences are almost identical each other and could be used as reference for downstream experiments.

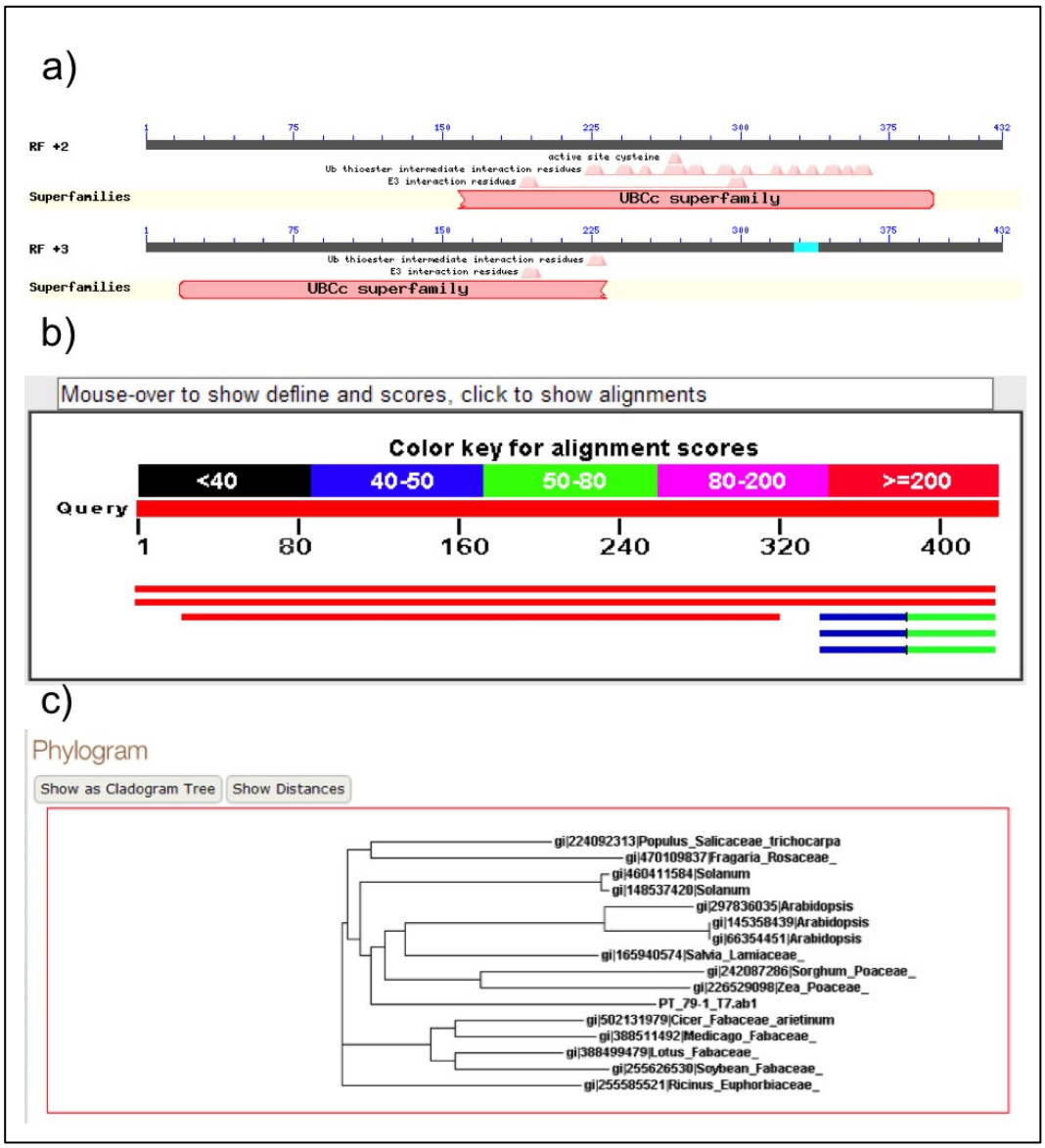

Figure 61 a)Protein domain search with BLASTx; b) Nucleotide BLASTn search; Phylogram of UBC gene family with the obtained sequences.

UBC 8-28 gene

Only *L. vulgare* cDNA template provided remarkable results. Both obtained sequences are specific to *UBC* domain, but a clear identification of the correspondence to *UBC_8* or *UBC_28* gene was not possible.

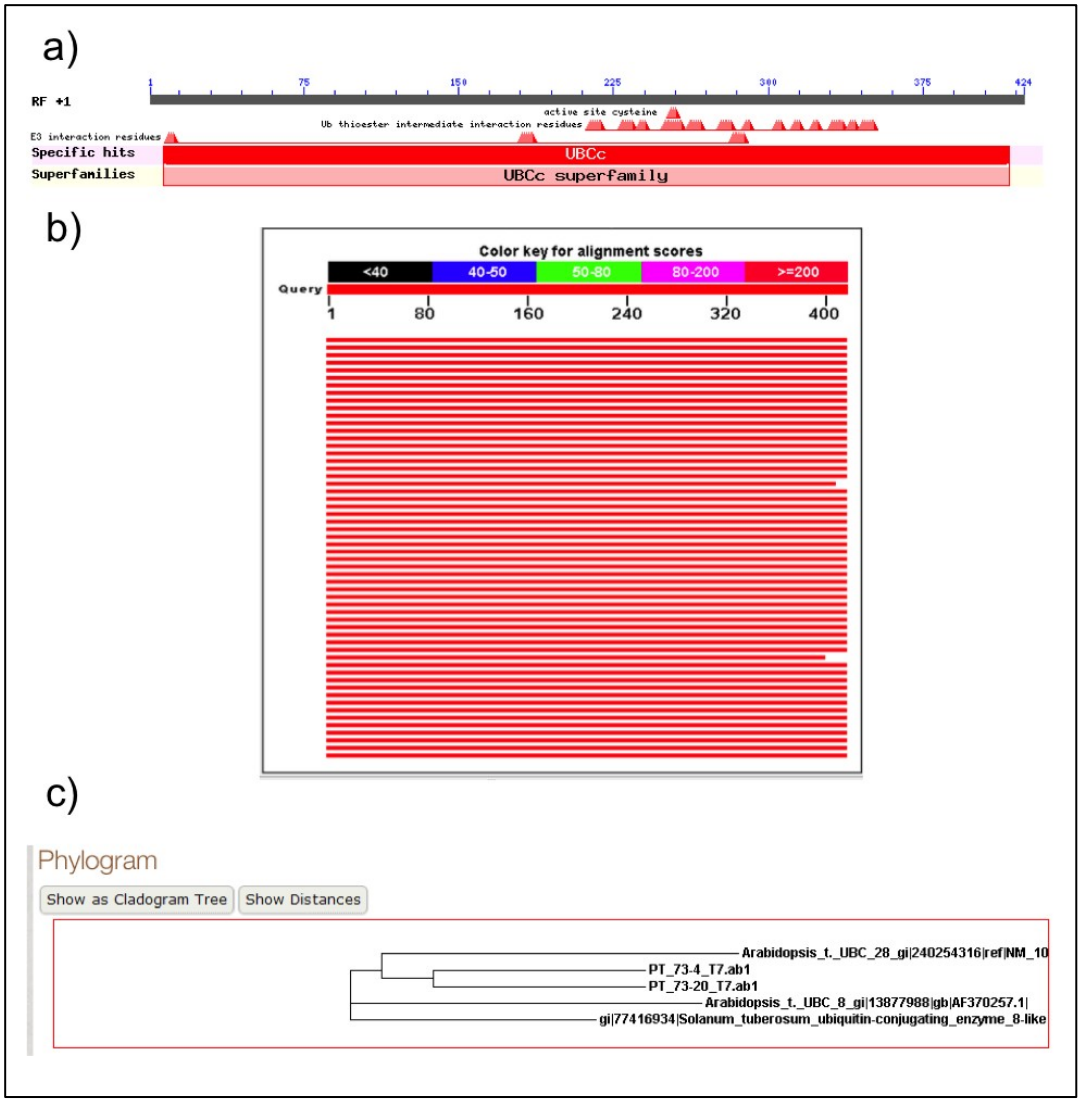

Figure 62 a)Protein domain search with BLASTx; b) Nucleotide BLASTn search; Phylogram of UBC gene family with the obtained sequences.

UBC 9-10 gene

For this gene all the obtained sequences were amplified from *L. vulgare* cDNA and they are very similar each other. In comparison with all the other *UBC* sequences, taken from NCBI database, they clustered together apart from other sequences.

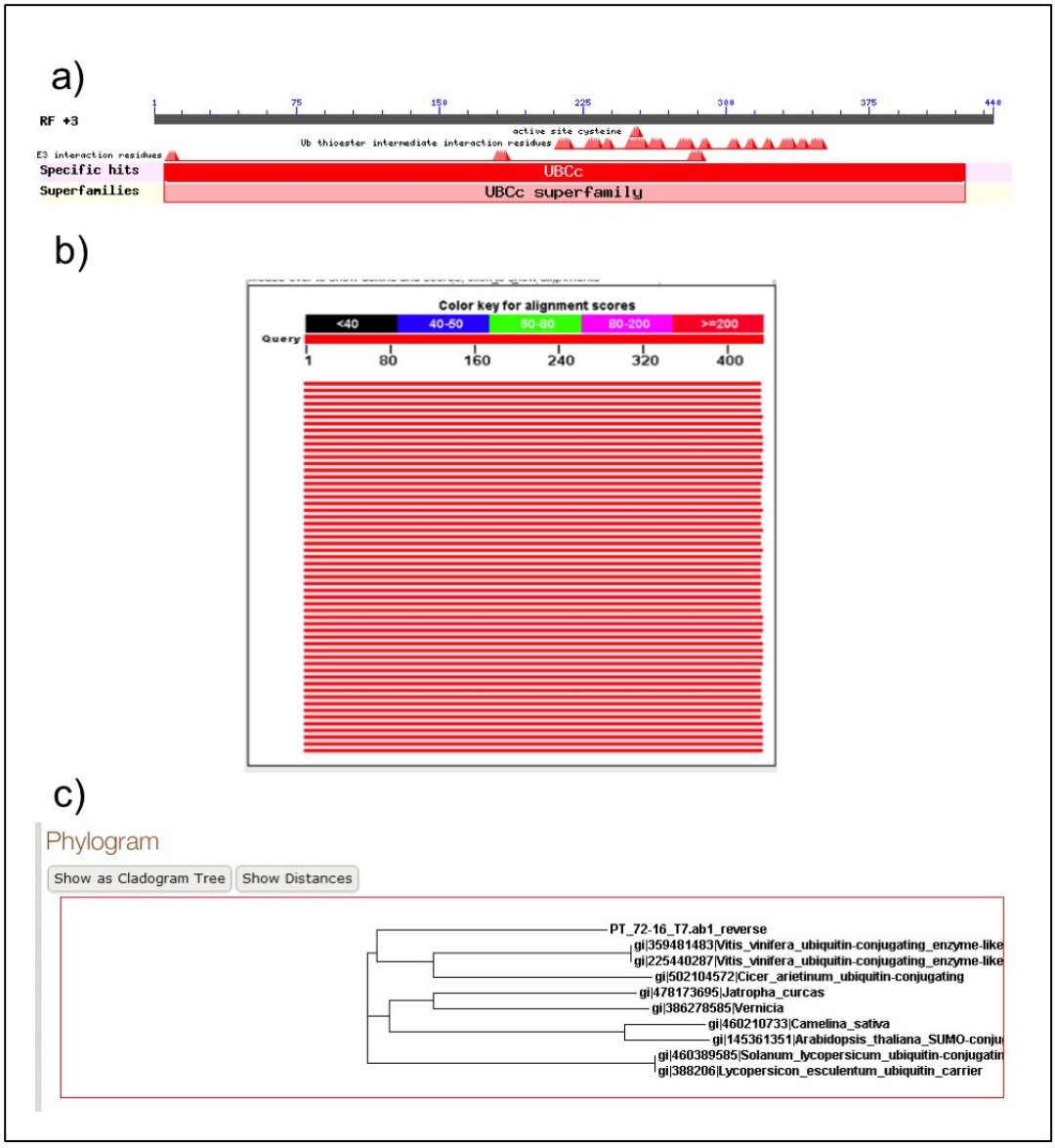

Figure 63 a) Protein domain search with BLASTx; b) Nucleotide BLASTn search; Phylogram of UBC gene family with the obtained sequences.

UBC sequences:

Only the amplicons obtained using *L. vulgare* cDNA as template gave significant results. In the following two phylograms (Figure 64) are reported which show that:

- PT_79 sequences are similar to *UBC_21* gene sequence as expected;
- PT_73 sequences are similar to *UBC_8* gene sequence and UBC 28 as expected;
- PT_72 sequences are similar to *UBC_9* and *UBC_10* gene sequences as expected.

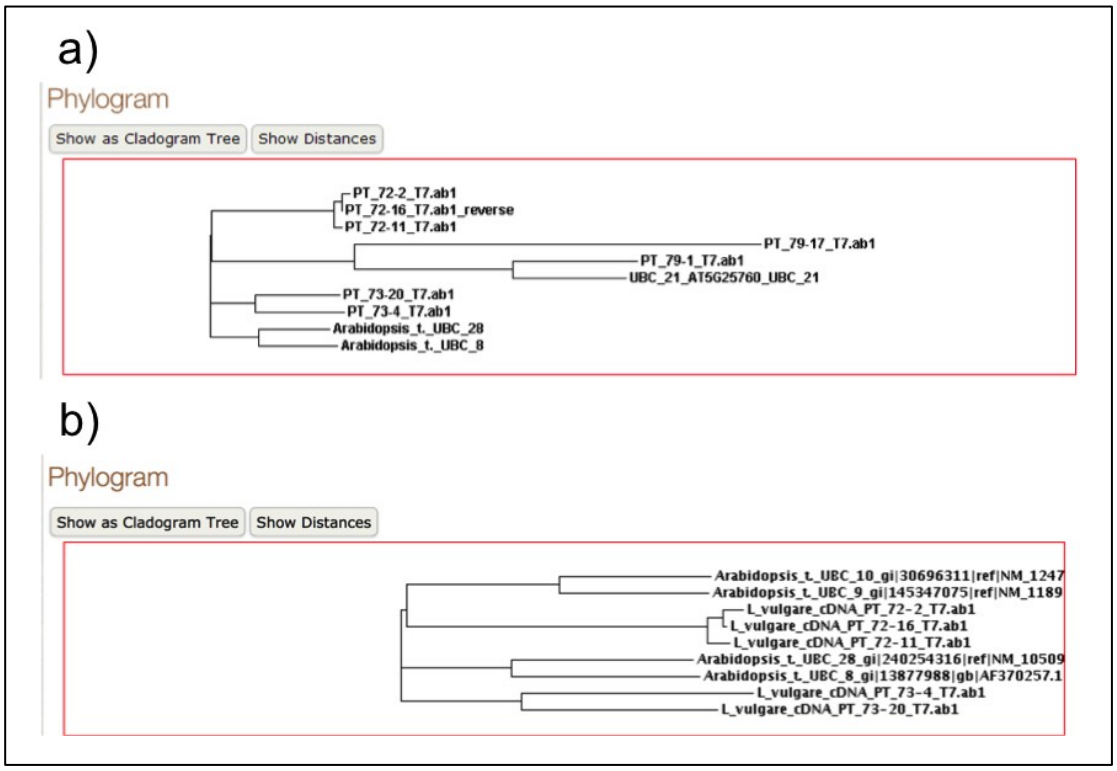

Figure 64 a) and b) phylograms of all the obtained UBC sequences compared with Arabidopsis sequences.

Expansin gene:

Both *C. coronarium* and *L. vulgare* sequences during BLASTx search matched *DPBB* domain, which is a conserved region that has the Double-Psi Beta-Barrel (DPBB) fold. Moreover another hit was the *PLN00050* domain, which is a provisional domain for *Expansin A*.

Sequences from both organism are similar each other. Alignment among *C. coronarium*, *L. vulgare* sequences and *Gerbera hybrid* and *Zinnia elegans* Expansin gene sequence showed that *C. coronarium* clustered apart from other sequences (Figure 65).

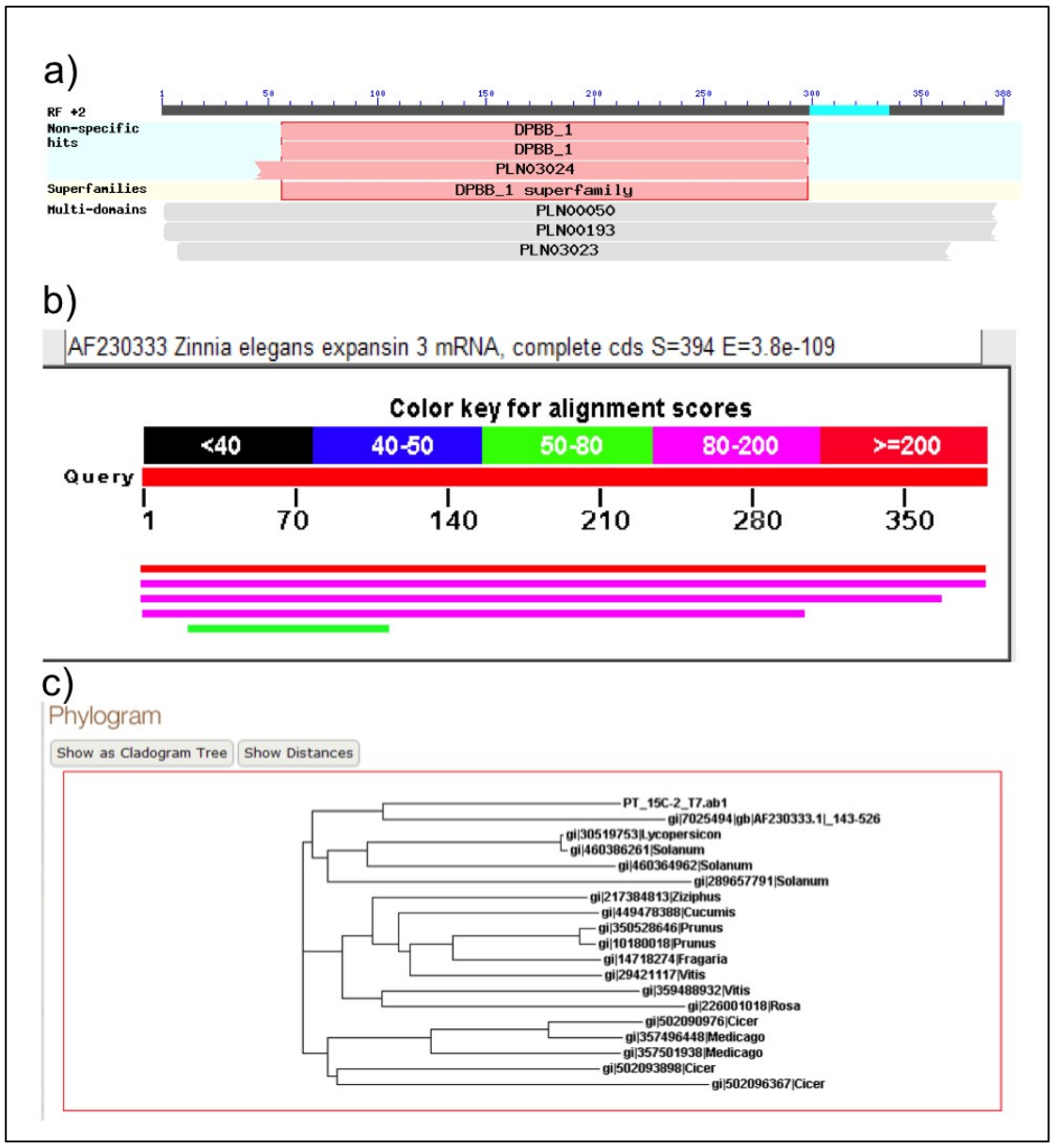

Figure 65 a)Protein domain search with BLASTx; b) Nucleotide BLASTn search; Phylogram of expansin gene family with the obtained sequences. GenBank: AF230333.1 is "*Zinnia elegans* expansin 3 mRNA, complete cds".

Discussion

Chrysanthemum coronarium germination physiology

Investigation on germination behaviour of *Chrysanthemum coronarium* point out that this weed species is dormant as suggested earlier (F. M. Bastida 2004) (Banon 2009) (F. J. Bastida 2010). Although excised seeds from pericarp tissues fully germinated, anatomical observation of pericarp tissue showed the presence of brachysclereid cells with multiple pit canals by which water can flow during achene imbibition. In addition, imbibition curve of both morphotype showed clearly that covering seed structures does not prevent water uptake. So that, a physical dormancy, which, according to Baskin & Baskin seed dormancy classification (J. M. Baskin 2004), corresponds to the presence of specialized structures that avoid water entrance, appears to be unlikely. On the other hand, investigation on physiological dormancy release through several methodologies does not provided any incontrovertible evidence of a complete dormancy breaking. However, eight-weeks wet-warm stratified achenes at 35/20° C produced a significant increase of germinability (up to 55%) for both varieties in central and in peripheral achenes. So, the synergistic presence of water together with alternating high temperature could play a role in dormancy release in *Chrysanthemum coronarium*.

Furthermore, achenes tested with dry-storage conditions showed a dormancy release (up to 35% of germination).

Interestingly, in all the tested conditions fruit coat rupture always preceded germination this event occurred in a much more faster way in wet-warm stratified seeds then in other conditions. Also long term dry storage achenes resulted in a high percentage of fruit coat rupture. This could be explained with the action that seed coat may have in mechanical constraint to the radicle protrusion, due to the hard lignified pericarp. In particular, in peripheral seed the broader thickness of pericarp could increase their inability to promptly germinate when imbibed.

The type of dormancy operating in *Chrysanthemum coronarium* best fits the model of non-deep physiological dormancy. In seeds with this type of dormancy, fresh seeds fail to germinate unless they receive a dormancy-breaking treatment of some kind (e.g. cold or warm temperatures, chemicals, or light). Excised embryos (or seeds) grow into normal seedlings however.

Inhibition of germination mediated by seed surrounding structure is widely reported in literature often due to presence of inhibitors (K. V. Beneke 1992) (K. V. Beneke 1993), or thick pericarp (Tanowitz 1987), or blocking diffusion of gasses (McEvoy 1984).
Thus, observing the 40 and 45° C wet-warm treatment, the tentative conclusion that the wet-warm cue may have rapidly weakened the fruit coat is very interesting, and worthy of follow-up. The reason of this weakening may lie in a seed over-imbibition allowed by a stronger seed osmotic potential. Seed osmotic potential increase may originate from dormancy-release associated physiological mechanisms or just by a physical mechanism where, according to van't Hoff equation, an increase in temperature cause a slightly increase in ψ_π value.
In natural environment a probable mechanism through which dormancy is released could be through pericarp tissue weaken which is achieved after long time in soil (more prolonged for peripheral achenes than central ones) establishing, eventually, a robust seed bank in the soil.

Chrysanthemum coronarium varietal genotyping

No significant differences were observed in germination behaviour between the two varieties. Molecular analyses are still underway and from preliminary observations there is not a clear differentiation between the two varieties either in ISSR profile or with barcode approach. If this interpretation will be confirmed an intra-specific differentiation between the two varieties would be more likely then an inter-specific one.

Leucanthemum vulgare germination physiology

Leucanthemum vulgare is a non-dormant species and it is very fast in germination within a broad range of temperatures. Interestingly, the priming treatment causes a germination response inhibition at low temperature that appear to be a *dormancy-induction* process. After this dormancy induction treatment seed germination is inhibited at 10° C and the germination temperature range is moved towards higher temperatures.

Using longer priming periods the dormancy state starts to weaken. In particular, after the long priming treatment (nine-weeks priming) at 5° C dormancy seems to be completely released.

Incubation in darkness could have a role in dormancy induction because seed primed in light especially at 10° produces a final germination percentage higher than seeds primed in dark with the same conditions. Low water potential is also involved in the germination inhibition causing a decrease of 29% when compared with control at 10° C.

Furthermore, giberellic acid added to 5 weeks primed seeds caused an increase of 20% in germination frequency.

On the other hand, fluridone addition during one or five-weeks (data not shown) priming treatment did not prevented the dormancy induction process. While when one-week primed seeds were imbibed with 50 μM of fluridone it produced a significant germination increase (about 40%) suggesting that, at this stage, ABA synthesis could have a role in dormancy maintenance.

This discrepancy in germinability in response to fluridone addition, could be explained supposing that the dormancy induction during priming is not an ABA mediated process, while, later, ABA mediates the maintenance of dormancy, as widely investigated in literature. Conversely, the dormancy induction process could be produced by a synergistic effect of low water osmotic potential and darkness.

Cloning of reference and dormancy associated genes:

Five probable housekeeping genes *18S* rRNA, *Phosphoglycerate kinase (PGK)* and *UBC_8*, *UBC_9*, *UBC_21* in *Leucanthemum vulgare* and in *Chysanthemum coronarium* var. *concolor* were selected, amplified, cloned and identified suitable for normalizing real-time PCR data. This is the first study to identify the appropriate reference genes in these two weed species. Subsequent examination of expression level of these isolated genes could validate as reliable genes for reference in quantitative PCR studies.

With the same methodology *Expansin* gene was isolated in *Leucanthemum vulgare* and in *Chrysanthemum coronarium* species. *DOG1*, *FLC* and *HUB2* genes isolation is underway. The expression profile of these genes will be analysed during different developmental stage of seeds, natural dormancy, and induction of dormancy. This will unveil their relationship with the molecular regulation of dormancy.

Conclusions

In this work the germination physiology of *Chrysanthemum coronarium* var. *concolor* and *discolor* was investigated and the condition for dormancy release were identified. This finding gives a substantial contribution to the knowledge of seed germination in *Chrysanthemum coronarium* for which relatively few studies are available. Further studies are required to assess the specific mechanism of dormancy release during warming treatment, how this is related to fruit coat weakening and if this would be associated to a change in seed osmotic potential.

Moreover, a system for dormancy induction and dormancy release was identified in a non-dormant species, *Leucanthemum vulgare*, which could be used as reference weed species in further comparative studies on dormancy regulation mechanisms.

In addition, isolation of reference genes to be used in quantitative PCR analysis together with the identification of dormancy-associated genes in these two species would be an essential tool for gene expression analysis of dormancy establishment and its maintenance.

Reference cited

- Amen, R.D. "A model of seed dormancy." *Botanicl Review* 34 (1968): 1-31.
- Amritphale, D., Dixit S., Singh B. "Effect of acetone on the induction and breakage of secondary dormancy in seeds of cucumber." *Journal of Experimental Botany* 44 (1993): 1621-1626.
- Amritphale, D., Sreenivasulu Y., Singh B. "Changes in Membrane Fluidity and Protein Composition during Release of Cucumber Seeds from Dormancy by a Higher Temperature Shift." *Annals of Botany* 85 (2000): 13-18.
- Arif, M., Jan M. T., Marwat K. B., Khan M. A. "Seed priming improves emergence and yield of soybean." *Pakistan Journal of Botany* 40, no. 3 (2008): 1169-1177.
- Ascher, P. "Self-incompatibility systems in floriculture crops." *Acta Horticulturae* 63 (1976): 205-216.
- Ashraf, M., Foolad R.M. "Pre-sowing seed treatment—a shotgun approach to improve germination, plant growth and crop yield under saline and non-saline conditions." *Advances in Agronomy* 88 (2005): 223-271.
- Augspurger, C.K. "Seed dispersal of the tropical tree Platypodium elegans and the escape of its seedlings from fungal pathogens." *Journal of Ecology* 71 (1983): 759-771.
- Banon, S., Martinez-Sanchez, J.J., Vicente, M.J., Conesa, E., Franco, J.A., Fernandez, J.A. "Effect of applying commercial gibberellins on seed germination of Chrysanthemum coronarium L." *Acta horticulturae* 813 (2009): 435-439.
- Barcaccia, G., F. Arzenton, T. F. Sharbel, S. Varotto, P. Parrini, and M. Lucchin. "Genetic diversity and reproductive biology in ecotypes of the facultative apomict Hypericum perforatum L." *Heredity* 96 (2006): 322–334.
- Baskin, C. C. "Breaking physical dormancy in seeds – focussing on the lens." *New Phytologist* 158, no. 2 (2003): 229-232.
- Baskin, C. C., J. M. Baskin. *Seeds: ecology biogeography and evolution of dormancy and germination.* San Diego: Academic Press, 1998.
- Baskin, C. C., J. M. Baskin, A. Yoshinaga, K. Thompson. "Germination of drupelets in multi-seeded drupes of the shrub Leptecophylla tameiameiae (Ericaceae) from Hawaii: a case for deep physiological dormancy broken by high temperatures." *Seed Science Research* 15, no. 4 (2005): 349-356.
- Baskin, J. M., C. C. Baskin. "A classification system for seed dormancy." *Seed Science Research* 14, no. 1 (2004): 1-16.
- Baskin, J. M., C. C. Baskin. "Effect of chilling and giberellic acid on growth potential of excised embryos of Ruellia humilis." *Planta* 100 (1971): 365-369.
- Baskin, J. M., C. C. Baskin. "Germination ecology of Phacelia dubia var. dubia in Tennessee glade." *American Journal of Botany* 58 (1971): 98-104.
- Baskin, J. M., C. C. Baskin. "Role of temperature in the germination ecology of three summer annual weeds." *Oecologia* 30 (1977): 377-382.
- Baskin, J. M., C. C. Baskin. "Temperature requirement for after-ripening in buried seeds of four summer annual weeds." *Weed Research* 27 (1987): 385-389.
- Baskin, J. M., C. C. Baskin. "Temperature requirements for afterripening of seeds of a winter annual induced into secondary dormancy by low winter temperatures." *Bulletin of the Torrey Botanical Club* 105 (1978): 104-107.

- Baskin, J. M., C. C. Baskin, L. Xiaojie. "Taxonomy, anatomy and evolution of physical dormancy in seeds." *Plant Species Biology* 15, no. 2 (2000): 139-152.
- Baskin, J. M., X. Nan, C. C. Baskin. "A comparative study of seed dormancy and germination in an annual and a perennial species of Senna (Fabaceae)." *Seed Science Research* 8 (1998): 501–512.
- Baskin, J.M., Baskin, C.C. "Germination dimorphism in Heterotheca subaxillaris var. subaxillaris." *Bulletin of the Torrey Botanical Club* 103 (1976): 201-206.
- Basra, S. M. A., Farooq M., Tabassum R. "Physiological and biochemical aspects of seed vigour enhancement treatments in fine rice (Oryza sativa L.)." *Seed Science Technology* 33 (2005): 623–628.
- Basra, S. M. A., Pannu I. A., Afzal I. "Evaluation of seedling vigor of hydro and matriprimed wheat (Triticum aestivum L.) seeds." *International Journal of Agricultural Biology* 5 (2003): 121-123.
- Bastida, F., J.L.G. Andujar, F. J. Monteagudo, J. Menendez. "Aerial seed bank dynamics and seedling emergence patterns in two annual Mediterranean Asteraceae." *Journal of Vegetation Science* 21 (2010): 541-550.
- Bastida, F., Menendez J. "Germination requirements and dispersal timing in two heterocarpic weedy Asteraceae." *Communications in Agricultural and Applied Biological Sciences* 69 (2004): 67-76.
- Beltran-Petia, E., A. Ortiz-López, E. Sanchez de Jiménez. "Synthesis of ribosomal proteins from stored mRNAs early in seed germination." *Plant Molecular Biology* 28 (1995): 327-336.
- Beneke, K., Van Rooyen M. W., Theron G. K. "Fruit polymorphism in ephemeral species of Namaqualand. IV. Growth analysis of plants cultivated from the dimorphic diaspores." *Journal of Arid Environments* 24 (1993): 345–360.
- Beneke, K., Von Teichman I., Van Rooyen M.W., Theron G.K. "Fruit polymorphism in ephemeral species of Namaqualand. I. Anatomic differences between polymorphic diaspores of two Dimorphotheca species." *South African Journal of Botany* 58 (1992): 448–455.
- Bennett, M. A., Waters L. "Seed hydration treatments for improved sweet maize germination and stand establishment." *Journal American Society Horticultural Science* 112 (1987): 45–49.
- Bentsink, L., Hanson J., Hanhart C. J., Vries H. B., C. Coltrane, P. Keizer, M. El-Lithyb, C. Alonso-Blancoe, M. T. de Andrése, M. Reymondf, F. vann Eeuwijk, S. Smeekens, and M. Koornneef. "Natural variation for seed dormancy in Arabidopsis is regulated by additive genetic and molecular pathways." *Proceedings of the National Academy of Sciences* 107, no. 9 (2010): 4264–4269.
- Bentsink, L., Jowett J., Hanhart J. C., Koornneef M. "Cloning of DOG1, a quantitative trait locus controlling seed dormancy in Arabidopsis." *PNAS* 103, no. 45 (2006): 17042–17047.
- Bewley, J. D. "Seed Germination and Dormancy." *The Plant Cell* 9 (1997): 1055-1066.
- Bewley, J. D., A. Marcus. "Gene expression in seed development and germination." *Progress in Nucleic Acid Research and Molecular Biology* 38 (1990): 165-193.
- Bewley, J. D., M. Black. *Seeds: Physiology of Development and Germination.* New York: Plenum Press, 1994.
- Bhattacharyya, D. "Karyomorphological studies in some species of the genus Chrysanthemum." *Science and Culture* 43 (1977): 431-432.

- Bhowmik, P. C. “Weed biology: importance to weed management.” *Weed science* 45 (1997): 349-356.
- Blaxter, M. L. “The promise of a DNA taxonomy.” *Philos Trans R Soc Lond B Biol Sci* 359, no. 1444 (2004): 669-679.
- Blumenthal, A., Lerner, H.R., Werker, E., Poljakoff-Mayber, A. “Germination preventing mechanisms in Iris seeds.” *Annals of Botany* 58 (1986): 551-561.
- Bornet, B., Branchard M. “Non anchored inter simple sequence repeat (ISSR) markers: reproducible and specific tools for genome fingerprinting.” *Plant Molecular Biology RepORT* 19 (2001): 209-215.
- Botha, F.C., Potgieter, G.P., Botha, A.M. “Respiratory metabolism and gene expression during seed germination.” *Journal of Plant Growth Regulation* 11 (1992): 211-224.
- Brändel, M. “Dormancy and germination of heteromorphic achenes of Bidens frondosa.” *Flora* 199 (2004): 228–233.
- Brändel, M. “Ecology of achene dimorphism in Leontodon saxatilis.” *Annals of Botany* 100 (2007): 1189–1197.
- Bradford, K. J. “Manipulation of seed water relations via osmotic priming to improve germination under stress conditions.” *Horticoltural Science* 21 (1986): 1105-1112.
- Bray, C. M., Davison P. A., Ashraf M., Taylor M. R. “Biochemical events during osmopriming of leek seed.” *Annals of Applied Biology* 102 (1989): 185-193.
- Bremer, K., Humphries, C. J. *The generic monograph of Asteraceae-Anthemideae.* . Vol. 23. London: Bull. Nat. History Mus., 1993.
- Bruggink, G. T., Ooms J.J.J., Vander Toorn P. “Induction of longevity in primed seeds.” *Seed Science Research* 9 (1999): 49-53.
- Brullo, S., Costanzo E., Tomaselli V. “Phytosociological study on the Laurus communities in the Hyblaean Montains (SE Sicily).” *Phytocoenologia* 31, no. 2 (2001): 249-170.
- Brullo, S., Marcenò C. “Contributo alla conoscenza della vegetazione nitrofila della Sicilia.” *Coll. Phytos.* 12 (1985): 23-148.
- Cadman, C.S.C., Toorop, P.E., Hilhorst, H.W.M., Finch-Savage, W.E. “Gene expression profiles of Arabidopsis Cvi seeds during dormancy cycling indicate a common underlying dormancy control mechanism.” *Plant Journal* 46 (2006): 805-822.
- Cano, E., C. M. Musarella, A. Cano-Ortiz, C. J. Pinto Gomes, G. Spampinato, G. Ferro, J. A. Garrido, J. Mota. “Analysis of the Chrysanthemum coronarium communities in the Mediterranean.” *Gestao e Conservacao da Biodiversidade.* Tortosendo, 2012. 56-57.
- Cano, E., C.M. Musarella, G. Spampinato, J. C. Piñar Fuentes, C. J.Pinto Gomes. “Taxonomic analysis of Glebionis coronaria (L.) Cass. ex Spach in the Mediterranean area.” *VII International Seminar Management and Biodiversity Conservation “Planning and management of agricultural and forestry resources”.* Gambarie d‟Aspromonte – RC (Italy),, 2013. 56.
- Cano-Ortiz, A., Cano, E., González, A. Cano-Ortiz, A. *Flora medicinal y aromática.* Malaga: Servicio de Publicaciones -Universidad de Jaén, 2007.
- Carpita, N. C., A. Sharia, J. P. Barnett, J. R. Dunlap. “Cold stratification and growth of radicles of loblolly pine (Pinus taeda) embryos.” *Physiologia Plantarum* 59 (1983): 601-606.
- Carr, G. D., R. M. King, A. M. Powell, H. Robinson. “Chromosome numbers in Compositae.” *American Journal of Botany* 86 (1999): 1003-1013.

- Caseiro, R., Bennett M. A., Marcos-Filho J. "Comparison of three priming techniques for onion seed lots differing in initial seed quality." *Seed Science Technology* 32 (2004): 365–375.
- Chen, F., Dahal P., Bradford K. J. "Two Tomato Expansin Genes Show Divergent Expression and Localization in Embryos during Seed Development and Germination." *Plant Physiology* 127, no. 3 (2001): 928–936.
- Chen, K., Arora R. "Dynamics of the antioxidant system during seed osmopriming, post-priming germination, and seedling establishment in spinach (Spinacia oleracea)." *Plant Science* 180 (2011): 212–220.
- Chen, S.S.C. "Germination of light inhibited seed of Nemophilia insignis." *American Journal of Botany* 55 (1968): 1177-1183.
- Chiang, G. C. K., Baruaa D., Kramera E. M., R. M. Amasinob, and K. Donohue. "Major flowering time gene, FLOWERING LOCUS C, regulates seed germination in Arabidopsis thaliana." *Proceedings of the National Academy of Sciences* 106, no. 28 (2009): 11661-11666.
- Chiang, M.H., Park, K.W. "Effects of temperature, light and mechanical treatment on seed germination of Chrysanthemum coronarium L." *Journal of Korean Society Horticultural Sciences* 35, no. 6 (1994): 534-539.
- Clavijo, E. Ruiz De. "The role of dimorphic achenes in the biology of the annual weed Leontodon longirrostris." *Weed research* 41 (2001): 275-286.
- Cockshull, K.E. "The photoperiodic induction of flowering in short-day plants." In *Light and the flowering process*, by Thomas B., Cockshull K.E. Prue V., 33-50. London: Academic Press , 1984.
- Comai, L., Harada, J.J. "Transcriptional activities in dry seed nuclei indicate the timing of the transition from embryogeny to germination." *Proceedings Natural Academy Science* 87 (1990): 2671-2674.
- Cook, R., Talley, T.S., Dayton, P., Wang, M., Nguyen, K. "The effect of invasive crown daisy (Chrysanthemum coronarium) on a coastal sage scrub arthropod community in Southern California." *California Invasive Plant Council Symposium.* 2011. 63-65.
- Corkid, L., Rincon, E., Vazquez-Yanes, C. "Effect of light and temperature on germination of heteromorphic achenes of Bidens odorata (Asteraceae)." *Canadian Journal of Botany* 69 (1991): 574–579.
- Cosgrove, D.J. "Relaxation in a high-stress environment: The molecular bases of extensible cell walls and cell enlargement." *Plant Cell* 9 (1997): 1031-1041.
- Crowe, J.H., L.M. Crowe. "1992). Membrane integrity in anhydrobiotic organisms: Toward a mechanism for stabilizing dry seeds." In *Water and Life*, by C.B. Osmond, and C.L. Bolis G.N. Somero, 87-103. Berlin: Springer-Verla, 1992.
- Cullings, K. W. "Design and testing of a plant-specific PCR primer for ecological and evolutionary studies." *Molecular Ecology* 1 (1992): 233-240.
- Czechowski, T., Stitt M., Altmann T., Udvardi M.K., Scheible W.R. "Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis." *Plant Physiology* 139, no. 1 (2005): 5-17.
- Dekkers, S.J.W., Willems L., Bassel G.W., van Bolderen-Veldkamp R.P., Ligterink W., Hilhorst H.W.M., and Bentsink L. "Identification of reference genes for RT-qPCR expression analysis in Arabidopsis and tomato seeds." *Plant Cell Physiology* 53 (2011): 28-37.
- d'Urville, Dumont. *Mémoire Société Linnéenne* 1 (1822): 368.

- Dogan, B., A. Duran, E. E. Hakki. "Phylogenetic analysis of Jurinea (Asteraceae) species from Turkey based on ISSR amplification." *Ann. Bot. Fennici* 44 (2007): 353-358.
- Dommes, J., C. Van de Walle. "Polysome formation and incorporation of new ribosomes into polysomes during germination of the embryonic axis of maize." *Physiologia Plantarum* 79 (1990): 289-296.
- Douglas, G. W., G. B. Straley, D. V. Meidinger, J. Pojar. *Illustrated Flora of British Columbia.* British Columbia, 1998.
- Dowrick, G.J. "The chromosomes of Chrysanthemum, I: The species." *Heredity* 6 (1952): 365-367.
- Doyle, J. J., and E. E. Dickson. "Preservation of plant samples for DNA restriction endonuclease analysis." *Taxon* 36 (1987): 715-722.
- Doyle, J. J., and J. L. Doyle. "A rapid DNA isolation procedure for small quantities of fresh leaf tissue." *Phytochemistry Bulletin* 19 (1987): 11-15.
- Ebach, M.C., Holdrege C. "DNA Barcoding is no substitute for taxonomy." *Nature* 434 (2005): 697.
- El-Twab A. M. H., Zahran F. A. "RAPD, ISSR and RFLP Analysis of Phylogenetic Relationships Among Congeneric Species (Anthemideae, Asteraceae) in Egypt." *International Journal of Botany* 6, no. 1 (2010): 1-10.
- El-Twab, M.H.A., Mekawy, A.M., El-Katatny, M.S. "Karyomorphological studies of some species of Chrysanthemum sensu lato in Egypt." *Chromosome Botany* 3 (2008): 41-47.
- Farooq, M., Basra S. M. A., Wahid A. "Priming of field sown rice seed enhances germination, seedling establishment, allometry and yield." *Plant Growth Regulation* 44 (2006): 285-294.
- Ferro, G. "La vegetazione di Butera (Sicilia Meridionale)." *Atti Ist. bot. Univ. Lab. critt. Pavia* 13 (1980): 51-118.
- Ferro, G. "Nuovi dati sulla flora e sulla vegetazione dei coltivi e degli incolti di Lipari (Isole Eolie)." *Quad. Bot. Amb. Appl.* 15 (2004): 21-39.
- Finch-Savage, W. E., C.S.C. Cadman, P. E. Toorop, J. R. Lynn, H. W. M. Hilhorst. "Seed dormancy release in Arabidopsis Cvi by dry after-ripening, low temperature, nitrate and light shows common quantitative patterns of gene expression directed by environmentally specific sensing." *Plant Journal* 51 (2007): 60-78.
- Finch-Savage, W. E., Dent K. C., Clark L.J. "Soak conditions and temperature following sowing influence the response of maize (Zea mays L.) seeds to on-farm priming (pre-sowing seed soak)." *Field Crops Research* 90 (2004): 361-374.
- Finch-Savage, W. E., J. H. W. Bergervoet, R. J. Bino, H. A. Clay, S. P. C. Groot. "Nuclear Replication Activity During Seed Development, Dormancy Breakage and Germination in Three Tree Species: Norway Maple (Acer platanoidesL.), Sycamore (Acer pseudoplatanusL.) and Cherry (Prunus aviumL.)." *Annals of Botany* 81, no. 4 (1998): 519-526.
- Finch-Savage, W. E., Leubner-Metzger G. "Seed dormancy and the control of germination." *New Phytologist* 171 (2006): 501-523.
- Finkelstein, R, Reeves, W, Ariizumi, T, Steber, C. "Molecular aspects of seed dormancy." *Annual Review Plant Biology* 59 (2008): 387-415.
- Footitt, S., I. Douterelo-Soler, H. Clay, and W. E. Finch-Savage. "Dormancy cycling in Arabidopsis seeds is controlled by seasonally distinct hormone-signaling pathways." *Proceedings of the National Academy of Sciences* 108, no. 50 (2011): 20236–20241.

- Forsyth C., Brown N.A.C.,. "Germination of the dimorphic fruits of Bidens pilosa ." *New Phytologist* 90 (1982): 151–164.
- Gallardo, K., C. Job, S. P.C. Groot, M. Puype, H. Demol, J. Vandekerckhove, and D. Job. "Proteomics of Arabidopsis seed germination: A comparative study of wild-type and gibberellin-deficient seeds." *Plant Physiology* 129 (2002): 823-837.
- Gutterman, Y. "Do germination mechanisms differ in plants originating in deserts receiving winter or summer rain?" *Israel Journal of Botany* 39 (1990): 355-372.
- —. *Seed Germination in Desert Plants.* Berlin, 1993.
- Han, B., D. W. Hughes, G. A. Galau, J. D. Bewley, A. R. Kermode. "Changes in late embryogenesis abundant (Lea) messenger RNAs and dehydrins during maturation and premature drying of Ricinus communis L. seeds." *Planta* 201 (1996): 27-35.
- Harms, K. E., S. J. Wright, O. Calderon, A. Hernandez, E. A. Herre. "Pervasive density-dependent recruitment enhances seedling diversity in a tropical forest." *Nature* 404 (2000): 493-494.
- Hilhorst, H.W.M., Finch-Savage, W.E., Buitink, J., Bolingue, W., Leubner-Metzger, G. *Dormancy in plant seeds.* Vol. 21, in *Dormancy and resistance in harsh environments*, by Lubzens E, 43-67. Topics in Current Genetics, 2010.
- Holdsworth, M. J., L. Bentsink, W. J. J. Soppe. "Molecular networks regulating Arabidopsis seed maturation, after-ripening, dormancy and germination." *New Phytologist* 179 (2008): 33-54.
- Hollingsworth, P. M, Forrest L. L., Spouge J. L., Mehrdad Hajibabaei, Sujeevan Ratnasingham, Michelle van der Bank, Mark W. Chase, Robyn S. Cowan, David L. Erickson, Aron J. Fazekas, Sean W. Graham, Karen E. James, Ki-Joong Kim, W. John Kress, Harald Schneider, Jonathan van AlphenStahl, Spencer C.H. Barrett, Cassio van den Berg, Diego Bogarin, Kevin S. Burgess, Kenneth M. Cameron, Mark Carine, Juliana Chacón, Alexandra Clark, James J. Clarkson, Ferozah Conrad, Dion S. Devey, Caroline S. Ford, Terry A.J. Hedderson, Michelle L. Hollingsworth, Brian C. Husband, Laura J. Kelly, Prasad R. Kesanakurti, Jung Sung Kim, Young-Dong Kim, Renaud Lahaye, Hae-Lim Lee, David G. Long, Santiago Madriñán, Olivier Maurin, Isabelle Meusnier, Steven G. Newmaster, Chong-Wook Park, Diana M. Percy, Gitte Petersen, James E. Richardson, Gerardo A. Salazar, Vincent Savolainen, Ole Seberg, Michael J. Wilkinson, Dong-Keun Yi, and Damon P. Little. "A DNA barcode for land plants." *Proceedings of the National Academy of Sciences* 106, no. 31 (2009): 12794-12797.
- Huangfu, C. h., X. l. Song, S. Qiang. "ISSR variation within and among wild Brassica juncea populations: implication for herbicide resistance evolution." *Genet Resour Crop Evol* 56 (2009): 913-924.
- Hwang, H.J. "Improvement of In Vitro Seed Germination in Chicory (Cichorium intybus L.) and Garland Chrysanthemum (Chrysanthemum coronarium L.)." *Journal of Bio-Environment Control*, 2008: 297-305.
- Imbert, E. "Ecological consequences and ontogeny of seed heteromorphism. Perspectives in Plant Ecology." *Perspectives in Plant Ecology, Evolution and Systematics* 5 (2002): 13-36.
- Imbert, E., Escarré J., Lepart J. "Differentiation among populations for life history, morphology, head traits and achene morph productions in the heterocarpic species Crepis sancta (Asteraceae)." *International Journal of Plant Sciences* 160 (1999): 543-552.

- Iqbal, M., Ashraf M. "Changes in growth, photosynthetic capacity and ionic relations in spring wheat (Triticum aestivum L.) due to presowing seed treatment with polyamines." *Plant Growth Regulation* 46 (2005): 19–30.
- Jain, A., Bhatia S., Banga S. S., Prakash S., Lakshmikumaran M. "Potential use of random amplified polymorphic DNA (RAPD) technique to study the genetic diversity in Indian mustard (Brassica juncea) and its relationship to heterosis." *Theoretical Applied Genetics* 88 (1994): 116–122.
- Janzen, D. H. "Seed predation by animals." *Annual review of Ecology and Systematics* 2 (1971): 465-492.
- Janzen, D. H., Hajibabaei M., Burns J. M., Hallwachs W., Remigio, E. and Hebert, P. D. N. "Wedding biodiversity inventory of a large and complex Lepidoptera fauna with DNA barcoding." *Philosophical Transactions of the Royal Society of London* 360 (2005): 1835-1845.
- Jauzein, P. *Flore des Champs Cultivés.* 2011.
- Jett, L. W., Welbaum G. E., Morse R. D. "Effect of matric and osmotic priming treatments on broccoli seed germination." *Journal of American Society Horticultural Science* 121 (1996): 423-429.
- Jiang, L., A. R. Kermode. "Role of desiccation in the termination of expression of genes for storage proteins." *Seed Science Research* 4 (1994): 149-173.
- Jisha, K. C., K. Vijayakumari, J. T. Puthur. "Seed priming for abiotic stress tolerance: an overview." *Acta Physiolia Plantarum* 35 (2013): 1381–1396.
- Juntilla, O. "The mechanism of low temperature dormancy in mature seeds of Syringa species." *Physiologia Plantarum* 29 (1973): 256-263.
- Kaul M.L., Kaur J. "Checkered prophase I in crown daisy." *Cytologia* 60 (1995): 195-203.
- Kaur, S., Gupta A. K., Kaur N. "Effect of osmo- and hydropriming of chickpea on seedling growth and carbohydrate metabolism under water deficit stress." *Plant Growth Regulation* 37 (2002): 17-22.
- Kaur, S., Gupta A. K., Kaur N. "Seed priming increases crop yield possibly by modulating enzymes of sucrose metabolism in chickpea." *Journal of Agronomical Crop Science* 19 (2005): 81-87.
- Kaya, M. D., Okcu G., Atak M., Cikili Y., Kolsarici O. "Seed treatments to overcome salt and drought stress during germination in sunflower (Helianthus annuus L.)." *European Journal of Agronomy* 24 (2006): 291-295.
- Keller, M., Kollmann J. "Germination under controlled and natural conditions." *Ecosystems and Environment* 72, no. 1 (1999): 87-99.
- Kendall, S. L., A. Hellwege, P. Marriot, C. Whalley, Graham IA, S. Penfield. "Induction of dormancy in Arabidopsis summer annuals requires parallel regulation of DOG1 and hormone metabolism by low temperature and CBF transcription factors." *Plant Cell* 23 (2011): 2568–2580.
- Khan, A. A., C. Samimy. "Hormones in relation to primary and secondary seed dormancy." In *The physiology and biochemistry of seed development*, by Khan A.A., 203-241. Amsterdam/New York/Oxford: Elsevier, 1982.
- Khuroo, A.A., Malik, A.H., Reshi, Z.A., Dar, G.H. "From ornamental to detrimental: plant invasion of Leucanthemum vulgare Lam. (Ox-eye Daisy) in Kashmir valley, India." *Current Science* 98, no. 5 (2010): 601-602.
- Kilian, J., D. Whitehead, J. Horak, D. Wanke, S. Weinl, O. Batistic, C. D'Angelo, E. Bornberg-Bauer, J. Kudla, K. Harter. "The AtGenExpress global stress expression data set: protocols, evaluation and model data analysis of UV-B light, drought and cold stress responses." *Plant Journal* 50 (2007): 347-363.

- Kocik, A. 1996.
- Koonjul, P. K., W. F. Brandt, J. M. Farrant, and G. G. Lindsey. "Inclusion of polyvinylpyrrolidone in the polymerase chain reaction reverses inhibitory effects of polyphenolic contamination of RNA." *Nucleic Acids Research* 27, no. 3 (1999): 915-916.
- Kucera, B., Cohn, M.A., Leubner-Metzger, G. "Plant hormone interactions during seed dormancy release and germination." *Seed Science Research* 15, no. 4 (2005): 281-307.
- Kumar, K., M. Muthamilarasan, M. Prasad. "Reference genes for quantitative Real-time PCR analysis in the model plant foxtail millet (Setaria italica L.) subjected to abiotic stress conditions." *Plant Cell Tissue and Organ Culture* , 2010.
- Lahaye, R., Van der Bank M., Bogarin D., Warner J., Pupulin F., Gigot G., Maurin O., Duthoit S., Barraclough T. G., Savolainen V. "DNA barcoding the floras of biodiversity hotspots." *Proceedings of the National Academy of Sciences* 105, no. 8 (2008): 2923–2928.
- Lalonde, L., J. D. Bewley. "Desiccation during germination and its effects on the messenger RNA population." *Experimental Botany* 37 (1986): 754-764.
- Lane, B.G. "Cellular desiccation and hydration: Developmentally regulated proteins, and the maturation and germination of seed embryos." *FASEB Journal* 5 (1991): 2893-2901.
- Larkin, M. A., Blackshields G., Brown N.P., Chenna R., McGettigan P.A., McWilliam H., Valentin F., Wallace I.M., Wilm A., Lopez R., Thompson J.D., Gibson T.J. and Higgins D.G. "ClustalW and ClustalX version 2." *Bioinformatics* 23, no. 21 (2007): 2947-2948.
- Lauber, K., G. Wagner. *Flora Helvetica.* Berna, 2001.
- Le Page-Degivry, M. T., P. Barthe, and G. Garello. "Involvement of Endogenous Abscisic Acid in Onset and Release of Helianthus annuus Embryo Dormancy." *Plant Physiology* 92 (1990): 1164-1168.
- Lee, C. R., Mitchell-Odds T. "Quantifying effects of environmental and geographical factors on patterns of genetic differentiation." *Molecular Ecology* 20, no. 22 (2011): 4631-4642.
- Lem, N.W., J.P. Williams,. "Desaturation of fatty acids associated with monogalactosyl diacylglycerol: The effect of SAN 6706 and SAN 9785." *Plant Physiology* 68 (1981): 944-949.
- Leubner-Metzger, G. "ß-1,3-Glucanase gene expression in low-hydrated seeds as a mechanism for dormancy release during tobacco after-ripening ." *The Plant Journal* 41 (2005): 133-145.
- Leubner-Metzger, G. "Functions and regulation of ß-1,3-glucanase during seed germination, dormancy release and after-ripening." *Seed Science Research* 13 (2003): 17-34.
- Levin S., D. Cohen, A. Hastings. "Dispersal strategy in patchy environments." *Theoretical and Population Biology* 26 (1984): 165-191.
- Linkies, A., Müller, K., Morris, K., Turečková, V., Wenk, M., Cadman, C.S.C., Corbineau, F., Strnad, M., Lynn, J.R., Finch-Savage, W.E., Leubner-Metzger, G. "Ethylene interacts with abscisic acid to regulate endosperm rupture during germination: A comparative approach using Lepidium sativum and Arabidopsis thaliana." *The Plant Cell* 21 (2009): 3803–3822.
- Liu, Y., Koornneef, M., Soppe, W.J. "The absence of histone H2B monoubiquitination in the Arabidopsis hub1 (rdo4) mutant reveals a role for chromatin remodeling in seed dormancy." *The Plant Cell* 19 (2007): 433-444.

- Magneschi, L., Kudahettigea, R.L., Alpi, A., Perata, P. "Expansin gene expression and anoxic coleoptile elongation in rice cultivars." *Journal of Plant Physiology* 166 (2009): 1576-1580.
- Manz, B., K. Müller, B. Kucera, F. Volke, G. Leubner-Metzger. "Water Uptake and Distribution in Germinating Tobacco Seeds Investigated in Vivo by Nuclear Magnetic Resonance Imaging." *Plant Physiology* 138 (2005): 1538-1551.
- McDonald. "Seed priming. ." In *Seed technology and its biological basis.*, by M., Bewley J. D. Black, 287–325. Sheffield: Sheffield Academic Press, 2000.
- McDonough, W. T. "Dormant and non-dormant seeds: similar germination responses when osmotically inhibited." *Nature* 214 (1967): 1147-1148.
- McDonough, W. T. "Germination polymorphism in Grindelia squarrosa." *Northwest Science* 49 (1975): 190–200.
- McEvoy, P.B. "Dormancy and dispersal in dimorphic achenes of tansy ragwort, Senecio jacobaea L. (Compositae)." *Oecologia* 61 (1984): 160–168.
- McQueen-Mason, S., D. J. Cosgrove. "Expansin mode of action on cell walls. Analysis of wall hydrolysis, stress relaxation, and binding." *Plant Physiologist* 107 (1995): 87-100.
- Meusel, H., E. Jäger, E. Weinert. *Comparative Chorology of the Central European Flora.* Jena: Gustav Fischer Verlag, 1965.
- Mukherjee, S. K., Sarkar A. K. "Cypselar morphology and anatomy in some members of the family Compositae – tribe Anthemideae." In *Proceedings of the national symposium of plant sciences in the nineties*, by R. D., Sen S. P., Samaddar KR, Sen U, Sarkar AK and Biswas AK Banerjee, 448-464. Kalyani: Department of Botany, Kalyani University, 1992.
- Mullen, R. T., J. E. King, D. J. Gifford. "Changes in mRNA populations during loblolly pine (Pinus taeda) seed stratification, germination and postgerminative growth." *Physiologia Plantarum* 97 (1996): 545-553.
- Nascimento, W. M., West S. H. "Priming and seed orientation affect emergence and seed coat adherence and seedling development of muskmelon transplants." *Horticultural Science* 33 (1998): 847–848.
- Negbi M., Tamari B. "Germination of chlorophyllous and achlorophyllous seeds of Salsola volkensii and Aellenia autrani." *Israel Journal of Botany* 12 (1963): 124–135.
- Nikolaeva, M.G. *Physiology of deep dormancy in seeds.* Israel Program for Scientific Translations, 1969.
- Nirmala A., Rao P. N. "Somatic chromosome morphology in Asteraceae." *Cell and Chromosome Research* 9 (1986): 26-27.
- Olmstead, R. G., Michaels, H. J., Scott, K. M., & Palmer, J. D. "Monophyly of the Asteridae and identification of their major lineages inferred from DNA sequences of rbcL." *Ann. Mo. Bot. Gard.* 79 (1992): 249-265.
- Osborne, D. J., L. L. Boubriak. "DNA and desiccation tolerance." *Seed Science Research* 4 (1994): 175-185.
- Parera, C. A., Cantliffe D. J. "Improved germination and modified imbibitions of shrunken-2 sweet corn by seed disinfection and solid matrix priming." *Journal of American Society Horticultural Science* 116 (1991): 942–945.
- Pavone, P., Terrasi, C.M., Zizza, A. "Chromosome number reports LXXII." *Taxon* 30 (1981): 695-696.
- Pemadasa, M.A., Lovell, P.H. "Factors controlling germination of some dune annuals." *Journal of Ecology* 63 (1975): 41–59.

- Pennisi, E. “Wanted: A Barcode for Plants.” *Science* 318, no. 5848 (2007): 190-191.
- Pignatti, S. *Flora d'Italia.* Bologna: Edagricole, 1982.
- Probert, R.J. “The Role of Temperature in the Regulation of Seed Dormancy and Germination.” In *Seeds: the ecology of regeneration in plant communities.*, by M. Fenner, 261-292. CABI, 2000.
- Qian, W., Ge S., Hong D. Y. “Genetic variation within and among populations of a wild rice Oryza granulata from China detected by RAPD and ISSR markers.” *Theoretical Applied Genetics* 102 (2001): 440–449.
- Rajjou, L., K. Gallardo, I. Debeaujon, J. Vandekerckhove, C. Job, D. Job. “The effect of alpha-amanitin on the Arabidopsis seed proteome highlights the distinct roles of stored and neosynthesized mRNAs during germination.” *Plant Physiology* 134 (2004): 1598–1613.
- Ratnasingham, S. and Hebert P. D. N. “BOLD: The Barcode of Life Data System.” *Molecular Ecology Notes* 7, no. 3 (2007): 355–364.
- Razaq Z.A., Khatoon S., Ali S.I. “A contribution to the chromosome numbers of Compositae from Pakistan.” *Pakistan Journal of Botany* 20 (1988): 177-189.
- Rocha, O.J. “The effects of achene heteromorphism on the dispersal capacity of Bidens pilosa.” *International Journal of Plant Sciences* 157 (1996): 316–322.
- Rodríguez, M. V., P. E. Toorop, and R. L. Benech-Arnold. *Challenges Facing Seed Banks and Agriculture in Relation to Seed Quality.* Vol. 773, in *Seed Dormancy, Methods in Molecular Biology*, by Kermode A. R., 17-40. Humana Press, 2011.
- Rouhi, H. R., Aboutalebian M. A., Sharif-Zadeh F. “Effects of hydro and osmopriming on drought stress tolerance during germination in four grass species.” *International Journal Agricultural Science* 1 (2011): 704-774.
- Rubinoff, D., Cameron S., Will. K. “A Genomic Perspective on the Shortcomings of Mitochondrial DNA for "Barcoding" Identification.” *Journal of Heredity* 97, no. 6 (2006): 581-594.
- Sánchez-Martinez, D., P. PuigdomBnech, M. Pagès. “Regulation of gene expression in developing Zea mays embryos. Protein synthesis during embryogenesis and early germination of maize.” *Plant Physiology* 82 (1986): 543-549.
- Schaefer, H., Hardy, O.J., Silva, L., Barraclough, T.G., Savolainen, V. *Ecology Letters* 14 (2011): 389–396.
- Scheibe, J., A. Lang. “Lettuce seed germination: Evidence for a reversible light induced increase in growth potential and for phytochrome mediation of low temperature effect.” *Plant Physiology* 40 (1965): 485-492.
- Schmid, M., T. S. Davison, S. R. Henz, U. J. Pape, M. Demar, M. Vingron, B. Schölkopf, D.Weigel, J. U. Lohmann. “A gene expression map of Arabidopsis thaliana development.” *Nat Genet.* 37, no. 5 (2005): 501-506.
- Schmittgen, T.D., Zakrajsek B.A. “Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR.” *J Biochem Biophys Methods* 46, no. 1-2 (2000): 69-81.
- Schopfer, P., Placy, C. “Control of seed germination by abscisic acid. Effect of embryo growth potential (minimum turgor pressure) and growth coefficient (cell wall extensibility) in Brassica napus L.” *Plant Physiology* 77 (1985): 676-686.
- Shih, C., Fu G. *Angiospermae Dicotyledoneae Compositae 3 Antheimdeae.* Peking: Science Press, 1983.
- Smissen, R. D., I. Breitwieser, J. M. Ward, P. A. McLenachan, and P. J. Lockhart. “Use of ISSR profiles and ITS-sequences to study the biogeography of alpine

cushion plants in the genus Raoulia (Asteraceae).” *Plant Syst. Evol* 239 (2003): 79-94.
- Strother, J. L., L. E. Watson. *Chromosome numbers in Compositae from Morocco and Spain.* Vol. 17, in *Documented chromosome numbers*, by Watson L.E. Strother J.L., 627-629. Sida, 1997.
- Tanowitz, B. D., Salopek P. F., Mahall B. E. “Differential germination of ray and disc achenes in Hemizonia increscens (Asteraceae).” *American Journal of Botany* 74 (1987): 303–312.
- Taylor, A. G., Harman G. E. “Concepts and technologies of selected seed treatments.” *Annals Review Phytopathology* 28 (1990): 321-329.
- Thomas, U. C., Varughese K., Thomas A., Sadanandan S. *Seed priming for increased vigour, viability and productivity of upland rice.* Vol. 4. 14 vols. Leisa , 2000.
- Thompson, K., Rabinowitz D. “Do Big Plants Have Big Seeds?” *The American Naturalist* (The University of Chicago Press) 133, no. 5 (1989): 722-728.
- Thompson, P. A. “Changes in germination responses of Silene secundiflora in relation to the climate of its habitat.” *Physiologia Plantarum* 23 (1970): 739-746.
- Toorop, P. E., Aelst A. C., Hilhorst H. W. M. “The second step of the biphasic endosperm cap weakening that mediates tomato (Lycopersicon esculentum) seed germination is under control of ABA.” *Journal of Experimental Botany* 51, no. 349 (2000): 1371-1379.
- Toorop, P. E., Barocco R. M., Engler G., Groot S. P. C., Hilhorst H. V. M. “Differentially expressed genes associated with dormancy or germination of Arabidopsis thaliana seeds.” *Planta* 221 (2005): 637-647.
- Turland, N.J. “Proposal to conserve the name Chrysanthemum coronarium (Compositae) with a conserved type.” *Taxon* 53, no. 4 (2004): 1072-1074.
- Vicente, M.J., Martínez Sánchez, J.J., Conesa, E., Cros, V., Franco, J.A. “Seed germination studies on Chrysanthemum coronarium.” *VII International Workshop on Seed Biology.* Salamanca, 2002.
- Vogt R., Oberprieler C. “Mediterranean chromosome number reports.” *Flora Mediterranea*, 1993: 338-340.
- Wan, C-Y, T. A. Wilkins. “A modified hot borate method significantly enhance the yield of high quality RNA from cotton (Gossypium hirsutum L.).” *Analytical Biochemistry* 223 (1994): 7-12.
- Watkins, J. T., D. J. Cantliffe. “Mechanical resistance of the seed coat and endosperm during germination of Capsicum annuum at low temperature.” *Plant Physiology* 72 (1983): 146-150.
- Will, K. W., Mishler B. D., Wheeler Q.D. “The perils of DNA barcoding and the need for integrative taxonomy.” *Systematic Biology* 54, no. 5 (2005): 844-851.
- Williams, J. G. K., Kubelik A. R., Livak K. J., Rafalski J.A., Tingey S.V. “DNA polymorphisms amplified by arbitrary primers are useful as genetic markers.” *Nucleic Acid Research* 18 (1990): 6531-6535.
- Winter, D., Vinegar B., H. Nahal, R. Ammar, G. V. Wilson, N. J. Provart. “An “Electronic Fluorescent Pictograph” Browser for Exploring and Analyzing Large-Scale Biological Data Sets.” *Plos One* 2, no. 8 (2007): e718.
- Wolff, K., Van Rijn J.P., Hofstra H. “RFLP analysis in chrysanthemum probe and primer development.” *Theoretical Applied Genetics* 88 (1994): 472-478.
- Wu, Y., W. G. Spollen, R. E. Sharp, P. R. Hetherington, S. C. Fry. “Root growth maintenance at low water potentials. lncreased activity of xyloglucan

endotransglycosylase and its possible regulation by abscisic acid." *Plant Physiology* 106 (1994): 607-615.

- Yagmur M., Kaydan D. "Alleviation of osmotic strength of water and salt in germination and seedling growth of triticale with seed priming treatments." *African Journal of Biotechnology* 7 (2008): 2156–2162.
- Zietkiewicz E., Rafalski A., Labuda D. "Genome fingerprinting by simple sequence repeat (SSR) – anchored polymerase chain reaction amplification." *Genomics* 20 (1994): :176–183.
- Zlatanova, J. S., P. V. Ivanov, L. M. Stoilov, K. V. Chimshirova, B. S. Stanchev. "DNA repair precedes replicative synthesis during early germination of maize." *Plant Molecular Biology* 10 (1987): 139-144.