Plant-derived smoke and temperature effects on seed germination of five *Helianthemum* (Cistaceae)

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Abstract

Plant-derived smoke promotes germination in Mediterranean-like environments, but its effect is unclear in the Mediterranean Cistaceae. This article investigates the role of smoke in the comparative germination ecology of five Helianthemum taxa. Laboratory germination experiments were conducted using seeds collected in the field and stored in a seed bank. All seeds were mechanically scarified prior to testing. Various pre-treatments with smoke solutions and gibberellins were applied, and seeds were incubated in three germination temperature regimes (30/20 ℃, 22/12 ℃, 14/4 ℃). In H. cantabricum, H. nummularium, H. oelandicum and H. urrielense germination was very high at the three temperatures, and no effect of smoke was detected. In H. tinetense germination was generally poor and a significant effect of smoke was found, but the effect size was small. The thermal niche of Helianthemum appears to follow an opportunistic strategy, being limited only by physical dormancy at the time of dispersal. H. tinetense follows a more conservative strategy, with physiological dormancy retarding germination. In the Mediterranean basin and surrounding areas, smoke appears to be one of a multitude of environmental signals controlling germination. The smoke effect might be more relevant in certain species such as H. tinetense.

Keywords: Atlantic; endemic; fire ecology; Mediterranean; physical dormancy; physiological dormancy

Nomenclature: Castroviejo et al. (1986–2015) Flora iberica

1. Introduction

In ecosystems subjected to frequent fire disturbances, heat and smoke are major signals for seed germination (Cushwa et al., 1968; Jefferson et al., 2014). Heat may break the seed coat of physically dormant seeds (Baskin and Baskin, 2014), allowing imbibition during the next rainfall (Herranz et al., 1998). Smoke, produced by burning vegetation, has an important role by itself (van Staden et al., 2000). It carries chemicals, e.g. karrikins (Flematti et al., 2004; Guo et al., 2013), which can overcome physiological dormancy (Baskin and Baskin, 2014). Germination promotion by smoke is common in Mediterranean-like climates where fire plays a central role, like the South African fynbos (Brown, 1993), the Californian chaparral (Keeley and Fotheringham, 1998) and Western Australia (Dixon et al., 1995). Although earlier studies failed to detect a smoke response in the Mediterranean basin (Keeley and Babr-Keeley, 1999; Reyes and Casal, 2006; Reyes and Trabaud, 2009; Rivas et al., 2006), recent work suggests that smoke also promotes germination in the flora of the Mediterranean and surrounding areas (Crosti et al., 2006; Keeley et al., 2011; Mojzes et al., 2015; Moreira et al., 2010; Paula et al., 2009; Pérez-Fernández and Rodríguez-Echeverría, 2003).

Cistaceae are largely linked to the Mediterranean environment (Watson and Dallwitz, 1992). Physical dormancy is a common feature of the family (Ferrandis et al., 1999; Thanos et al., 1992); and post-fire seedling emergence has often been reported (Baskin and Baskin, 2014). A response to smoke has only been found in some species of genera such as Cistus and Fumana (Chen, 2014; Jefferson et al., 2014). In the diverse Helianthemum Mill. genus, heat breaks physical dormancy (Pérez-García and González-Benito, 2006), but attempts to find a germination response to smoke have been unsuccessful so far (Moreira et al., 2010). Five Helianthemum taxa occur in the Cantabrian Mountains (Carlón, 1998; López González, 1993). Seeds of these Cantabrian Helianthemum germinate massively after coat scarification, with the notable exception of the endemic H. tinetense, whose germination percentages are generally low. The special ecology of H. tinetense, closely linked to frequently burnt slopes (Carlón, 1998), makes it a good candidate to find a response to smoke (Mojzes et al., 2015). If such response exists in H. tinetense, it could be interpreted as one of the traits that allowed it to colonize its highly disturbed niche.

Thus, characterizing the germination response to smoke in these closely related Helianthemum can help to clarify the role of this signal in the functional ecology of the

Mediterranean Cistaceae. For this reason, we conducted a comparative seed germination study with five taxa of Helianthemum, focusing on H. tinetense. It was our goal to test the hypothesis that, in these taxa, and especially in H. tinetense, smoke promotes seed germination.

2. Materials and methods

2.1. Plant material

In this work we studied the germination of the five Helianthemum (Cistaceae) which occur in the Cantabrian Mountains: Helianthemum apenninum subsp. cantabricum (M. Laínz) G. López; Helianthemum apenninum subsp. urrielense (M. Laínz) G. López; Helianthemum nummularium (L.) Mill.; Helianthemum oelandicum subsp. incanum (Willk.) G. López; and Helianthemum tinetense Mayor & Fern. Benito. All taxa occupy dry scrub and grasslands at different altitudes. H. tinetense is the only one exclusive of acidic poor soils, usually in fire-prone slopes and road edges (Carlón, 1998).

We used seeds from two sources. First, to compare the germination of the five taxa, we used seeds that had been banked in the Seed Bank of the Jardín Botánico Atlántico (Xixón, Spain). All seed accessions had been collected in natural populations of the Cantabrian Mountains (Supplementary material 1). They had been preserved by drying to 15% RH and freezing at -13 °C (ENSCONET, 2009) and then stored for different time periods (10 to 1 years depending on the taxon, Supplementary material 1). Twenty-four h before the experiments began, we removed seeds from the storage freezers and allowed them to equilibrate to ambient temperature and humidity.

Second, to analyse germination of fresh seeds of H. tinetense, during August 2014 we collected ripe capsules from three natural populations: (1) Tinéu (43° 17' 12" N, 06° 31' 22" W ; 500 m above sea level); (2) Ayande (43° 16' 11" N, 06° 35' 30" W; 650 m); and (3) Ibias (43° 02' 25" N, 06° 51' 59" W; 300 m). In each site, we sampled all individuals bearing mature capsules. Capsules stayed in our laboratory (c. 21 °C, 50% RH) for two weeks before the start of germination experiments.

To produce a plant-derived smoke solution, we also collected plant remains from the Tinéu population, belonging to the dominant shrubs at the site: Calluna vulgaris, Cytisus striatus and Erica cinerea. We burnt these remains in controlled conditions, and during 5 minutes we impregnated the smoke produced by the combustion into (1) filter paper held 1 m above the fire (henceforth paper-smoke) and (2) an aerosol of distilled water passed through the smoke column (henceforth water-smoke).

2.2. Germination experiments

2.2.1. General conditions

To break the seed coat and remove the well-known physical dormancy of Helianthemum (Pérez-García and González-Benito, 2006; Baskin and Baskin, 2014; Royal Botanic Gardens Kew, 2016), we scarified all seeds with sandpaper before any further manipulation. Afterwards, we exposed seeds to one of four pre-treatments: (1) paper-smoke, 24 h immersion in 40 mL of distilled water with four 6 cm discs of paper-smoke, prepared as described above; (2) water-smoke, 24 h immersion in 30 mL of distilled water mixed with 10 mL of water-smoke, prepared as described above; (3) GA3, 24 h immersion in 40 mL of distilled water with gibberellic acid GA3 (0.5 g/L); and (4) control, 24 h immersion in 40 mL of pure distilled water. The purpose of the GA3 application was to confirm whether the low germination in H. tinetense was due to physiological dormancy. Then we sowed seeds in 6 cm Petri dishes holding 1% agar, and sealed the dishes with Parafilm to prevent agar from drying. We sowed 4 dishes with 25 seeds each per experimental treatment.

We incubated these dishes at one of three alternating temperature regimes programmed inside growth chambers (GROW-360, Ing Climas, Barcelona): (a) 30/20 ℃, a situation of soil heating; (b) 22/12 ℃, typical summer temperatures; and (c) 14/4 ℃, typical autumn or spring temperatures. Temperature regimes were coupled to a photoperiod of 12 h of light (c. 20 µmol m-2 s-1 produced by six fluorescent tubes) followed by 12 h of darkness. Incubations lasted 28 days, during which we scored germination daily, as radicle emergence. After the incubations, we opened non-germinated seeds with a scalpel and classified them as apparently normal, non-scarified (i.e., hard and not imbibed), empty and contaminated by fungi. We removed non-scarified, empty and infected seeds from further calculations. Thus, we considered that the number of potentially germinable seeds in each dish was the sum of germinated and non-germinated but apparently normal seeds. The cumulative number of germinated seeds in each scoring day, and the potentially germinable seeds in each dish, are provided in Supplementary material 2.

2.2.2. Comparative effect of temperature and smoke in Helianthemum

To compare the effect of temperature in the five taxa, and to test if the low germination of H. tinetense was due to a non-optimal germination temperature being used, we incubated non-treated (control) seeds at 30/20 ℃, 22/12 ℃ and 14/4 ℃. We sowed both non-stratified (sown directly) and cold-stratified seeds (previous 12 weeks at 3 °C in darkness and 1% agar), in order to assess the potential break of physiological dormancy by overwintering. However, in the end the effect of cold stratification could not be considered, because all taxa germinated during stratification (i.e. at 3 °C in darkness), with similar percentages to those of non-stratified seeds. In addition, to compare the germination response to smoke in the five taxa, we sowed seeds pre-treated with water-smoke at 22/12 ℃.

2.2.3. Effect of temperature and smoke in H. tinetense

For H. tinetense, we performed a factorial experiment to analyse the effect of smoke and temperature on the

germination of fresh seeds from the three natural populations. In this experiment we incubated control, paper-smoke, water-smoke and GA3 treated seeds at 30/20 ℃, 22/12 ℃ and 14/4 ℃.

2.3. Statistical analyses

We calculated statistics using R (R Development Core Team, 2008). We analysed the effect of the different experimental treatments on the final germination percentage (germinated / potentially germinable) after 28 days of incubation. For this we used Generalised Linear Models (GLM) with binomial error distribution and logit link function. In some treatments that had zero variance because all seeds germinated during the experiment, we subtracted one germinated seed from the fourth Petri dish. We began by fitting three fully factorial models to analyse (a) the effect of taxon and temperature in the genus; (b) the effect of taxon and smoke in the genus; and (c) the effect of population, temperature and smoke in H. tinetense fresh seeds. Afterwards, we performed a stepwise deletion of non-significant terms to produce a minimal adequate model for each case (Crawley, 2013). Then we assessed the significance of different experimental treatments by performing contrasts against a priori selected control treatments (i.e., the 22/12 °C germination temperature, the control smoke treatment, H. tinetense for the genus-level models, and the H. tinetense population with intermediate germination as assessed by visual inspection of the results for the H. tinetense models). For those contrasts that were significant, we calculated the estimated mean and the 95 % confidence intervals (CI) using the package 'effects' (Fox, 2003). CI are henceforth reported within brackets. Finally, to further visualize differences in the germination processes, we drew the germination progress curves of each treatment by selecting and fitting an appropriate growth function to the cumulative germination data, using the 'drc' package (Ritz and Streibig, 2005).

3. Results

3.1. Comparative effect of temperature and smoke in Helianthemum

A minimal adequate model for the effect of temperature in the final germination percentages of banked seeds of the five taxa included only the main effects of taxon and temperature (Table 1). Banked H. tinetense seeds had very low germination across temperature, 10.7% [7.5 – 15.0]. H. cantabricum, H. nummularium and H. urrielense formed a group with significantly higher germination across temperatures, 96.2% [94.7 – 97.3]. Finally, H. oelandicum was significantly different from the other Helianthemum, with 100% germination at the three temperatures. The contrasts for temperature indicated that germination was equally high at 14/4 °C and 22/12 °C (94.0% [91.4 – 95.9]), with a significant decrease at 30/20 °C (86.2% [80.3 – 90.6]). The comparison of the germination progress curves (Figure 1) highlighted the very fast and complete germination in H. oelandicum at all temperatures, as well as the poorer

results in H. tinetense. For the other three taxa, germination at 30/20 °C was remarkably slow compared to the other temperatures, even if the final germination was similar.

Regarding the effect of smoke on the seed germination of the five taxa at 22/12 °C, the minimal adequate model included the effects of taxon and smoke and their interaction (Table 1). This was mainly due to the significant interaction in H. nummularium, where germination decreased from 98.9% [93.1 – 99.9] in the control to 84.5% [75.1 – 90.7] in the smoke treatment. In H. cantabricum, H. nummularium, H. oelandicum and H. urrielense smoke did not affect the high germination percentages (98.5% [97.0, 99.2]). In H. tinetense, smoke-treated seeds had a higher average germination (20.0% [12.7 – 30.1]) than the control (10.3% [5.4 - 18.7]); but this difference was not significant (LR χ2 = 3.076, p = 0.079). The comparison of the germination progress curves (Figure 1) suggests that in H. cantabricum and especially in H. urrielense, smoke produced a slight acceleration of germination. Germination of smoke-treated seeds of H. tinetense at 22/12 °C was faster and higher than the germination of control seeds at any of the three temperatures (Figure 1).

3.2. Effect of temperature and smoke in H. tinetense

The minimal adequate model for the final germination percentages of fresh H. tinetense seeds included the effects of population, germination temperature and smoke, as well as the interaction between temperature and population (Table 1). The effect of germination temperature depended on the population, as 14/4 °C produced the highest germination percentages in seeds from Tinéu and Ayande, but significantly decreased germination in seeds from Ibias (Figure 2). The contrasts for the population effect indicated significantly more germination across treatments in seeds from Tinéu (83.5% [82.1 – 85.6]) than in a group formed by Ayande and Ibias (56.8% [54.3 – 59.2]). It must be noted that these fresh seeds germinated more than the banked seeds of H. tinetense (10.7% [7.5 – 15.0]), which had been collected the previous year from a different population, and whose fresh germination had been even lower (1%, Supplementary material 1). The contrasts for the smoke treatments created two groups, with significantly lower germination in the control and water-smoke with respect to paper-smoke and GA3, but the effect sizes were small (64.2% [61.4 – 66.9] vs. 70.0% [67.3 – 72.6.5]).

The comparison of the germination progress curves (Figure 2) indicates that germination tended to be faster at 22/12 °C than at either lower or higher temperatures, with steeper slopes at the beginning of the process. The germination progress curves also highlighted the inconsistency of the smoke effect on germination. The control tended to lag behind the other treatments especially at 30/20 °C and in the Ibias population. However, the opposite was also true in some cases such as Ayande seeds at 22/12 °C; and in several combinations

of population and temperature the germination progress in the control matched that in the smoke treatments.

4. Discussion

This study shows that Helianthemum germination is defined by a wide thermal niche and a high speed, and also suggests a limited physiological response to smoke. To our knowledge, this is the first report of germination response to smoke in the genus, although it had been found in other Cistaceae (Chen, 2014; Jefferson et al., 2014). Traditionally, it was considered that the flora of the Mediterranean basin differs from other Mediterranean-like floras in that heat only – and not smoke – drives the germination response to fire (Buhk and Hensen, 2006; Keeley and Babr-Keeley, 1999). This view has been challenged in recent studies (Keeley et al., 2011; Paula et al., 2009). In the Mediterranean flora, smoke seems to have a role in promoting the germination of species that do not fully respond to physical dormancy break (Moreira et al., 2010). This could be the case in H. tinetense, but the effect size is so small that it must be interpreted with caution.

We have determined that the optimum temperature for germination in the Cantabrian Helianthemum is around 10–15 ℃, which is consistent with previous studies (Escudero et al., 1997; Pérez-García and González-Benito, 2006; Pérez-García et al., 1995). However, the range of germination temperatures is remarkably wide, with similar results between 3 ℃ and 30/20 ℃. The ability to germinate at 3 ℃ in darkness suggests that seeds would germinate even under the cover of snow. As field temperatures at the collection sites are almost always within these values, seeds would germinate quickly as soon as the coat breaks and allows water imbibition. Moreover, in addition to a wide germination temperature range, our results show that seeds germinate very fast once physical dormancy has been broken. This phenomenon is generally associated with highly stressful habitats (Gutterman, 2002; Parsons, 2012), where seeds must seize an ephemeral establishment environment. Therefore, the germination of Helianthemum appears to be limited only by the availability of water in the soil and the permeability of the seed coat. The permeability of the seed coat can be regulated either by summer heat cycles or by fire-associated heat (Moreira and Pausas, 2012). Species in which physical dormancy can be broken by summer heat cycles are less bound to fire and can germinate during inter-fire gaps ('facultative pyrogenic dormancy'; Ooi et al., 2014). On the other hand, species in which physical dormancy is only broken by fire-related heat are tightly associated to post-fire germination ('obligate pyrogenic dormancy'; Ooi et al., 2014). As we did not investigate the thermal thresholds for physical dormancy break in our species, we cannot determine whether they fall in the facultative or obligate category.

Nevertheless, in species with pyrogenic dormancy, non-germination of a fraction of the seed population even after heat-related coat break is interpreted as bet-hedging

(Ooi et al., 2014). This seems to be the case of H. tinetense, which was the only studied taxon with consistently low germination percentages in the different tested populations. Of the five studied taxa, the ecology of H. tinetense, linked to disturbed and often burnt slopes (Carlón, 1998), made it a good candidate to respond to smoke (Mojzes et al., 2015). While it is true that the size of this effect was relatively small, it is clear that H. tinetense separates from the general germination pattern of the genus. In this regard, it must be noted that germination increased in response to GA3. The role of gibberellins as promoters of germination through suppression of physiological dormancy is well known (Bewley et al., 2013). Therefore, we can conclude that low germination in H. tinetense is due to a component of physiological dormancy, which joins physical dormancy, and seems to be partially overcome by smoke. Further studies would be needed to investigate the role of smoke in breaking this physiological dormancy. In our experiments, the paper-smoke solution produced a larger effect than water-smoke. This suggests that the concentration of plant-derived smoke compounds could vary between the two solutions, and different concentrations could elicit different germination responses. Therefore, it would be necessary to study the germination of H. tinetense in a more comprehensive range of smoke compounds and concentrations. It would also be worthy to explore further the apparent germination decrease as a response to smoke in H. nummularium, to determine if it is a physiological response or an artefact of the experiments.

In addition, future experiments must clarify the puzzling differences we found between populations. Germination was relatively high in the Tinéu seeds, compared to Ayande and Ibias, and especially low in the seeds obtained from the seed bank and that belonged to a fourth population from Cangas. The low germination of the banked seeds cannot be due to the conservation process, as the initial germination just after collection was even lower (Supplementary material 1). As these seeds were collected in a different site and year, the differences in germination could be due to local adaptation or phenotypic variation in physiological seed dormancy (Fernández-Pascual and Jiménez-Alfaro, 2014; Fernández-Pascual et al., 2013). In any case, it is interesting to note that the size of the smoke effect seemed to be larger in those populations that germinated less (Cangas and Ibias), supporting its potential role as a dormancy-breaking signal.

This study has provided some evidence for a physiological role of smoke in the germination of certain Helianthemum species. The effect appears to be subtle and is masked by the fast germination of the genus. Therefore, it is unclear whether this small effect does have an effect in the regeneration ecology of Helianthemum in the field. Given the reduced response we found, further field experiments are needed to test the relevance of smoke in ecologically-relevant situations. However, the physiological response we describe further

supports the role of smoke in controlling seed germination in the Cistaceae. Instead of a single major controller of seed germination, it seems to be one of a number of environmental signals (Crosti et al., 2006; Moreira et al., 2010).

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Table 1 Minimal adequate models fitted to the results of the germination experiments: comparative effect of temperature in the genus, comparative effect of smoke in the genus, and effect of temperature and smoke in fresh Helianthemum tinetense seeds.

Model	Parameter	Estimate	S.E.	z	p
Comparative temperature	Intercept (*H. tinetense* at 14/4 + 22/12 ℃)	-1.813	0.199	-9.104	<0.001
	Taxon (*H. cantabricum + H. nummularium + H. urrielense*)	5.359	0.275	19.490	<0.001
	Taxon (*H. oelandicum*)	6.788	0.620	10.948	<0.001
	Temperature (30/20 ℃)	-0.926	0.275	-3.364	<0.001
Comparative smoke	Intercept (*H. tinetense*, control)	-2.160	0.352	-6.134	<0.001
	Taxon (*H. cantabricum + H. oelandicum + H. urrielense*)	6.724	0.679	9.906	<0.001
	Taxon (*H. nummularium*)	6.7450	1.065	6.333	<0.001
	Smoke (Water-smoke)	0.773	0.450	1.720	0.085
	Taxon (*H. can. + H. oel. + H. urr.*) * Smoke (Water-smoke)	-1.550	0.842	-1.840	0.064
	Taxon (*H. num.*) * Smoke (Water-smoke)	-3.660	1.142	-3.206	0.001
H. tinetense fresh seeds	Intercept (Ayande at 22/12 ℃ with control or water-smoke)	0.170	0.120	1.426	0.154
	Smoke (Paper-smoke + GA$_3$)	0.264	0.088	3.012	0.003
	Temperature (14/4 ℃)	0.330	0.163	2.027	0.043
	Temperature (30/20 ℃)	-0.106	0.164	-0.656	0.512
	Population (Ibias)	0.347	0.184	1.890	0.059
	Population (Tinéu)	1.394	0.182	7.681	<0.001
	Population (Ibias) * Temperature (14/4 ℃)	-1.227	0.256	-4.786	<0.001
	Population (Ibias) * Temperature (30/20 ℃)	-0.492	0.260	-1.893	0.058
	Population (Tinéu) * Temperature (14/4 ℃)	-0.342	0.260	-1.317	0.188
	Population (Tinéu) * Temperature (30/20 ℃)	-0.113	0.260	-0.434	0.665
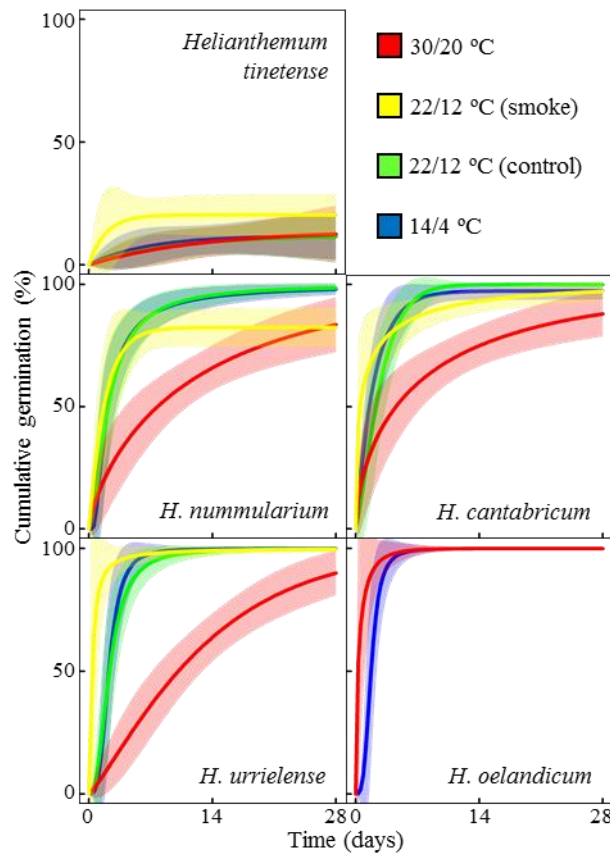
Figure 1 Germination progress of banked seeds from five Helianthemum taxa. Seeds were germinated at three temperature regimes, and seeds at 22/12 ºC were also pre-treated with plant-derived smoke (water-smoke). Darker curves are growth functions fitted to data using the package 'drc'. In each treatment, a best fit function was selected between the log-logistic, asymptotic regression and Weibull. Lighter areas are the 95 % confidence interval of each curve. Colour online only.
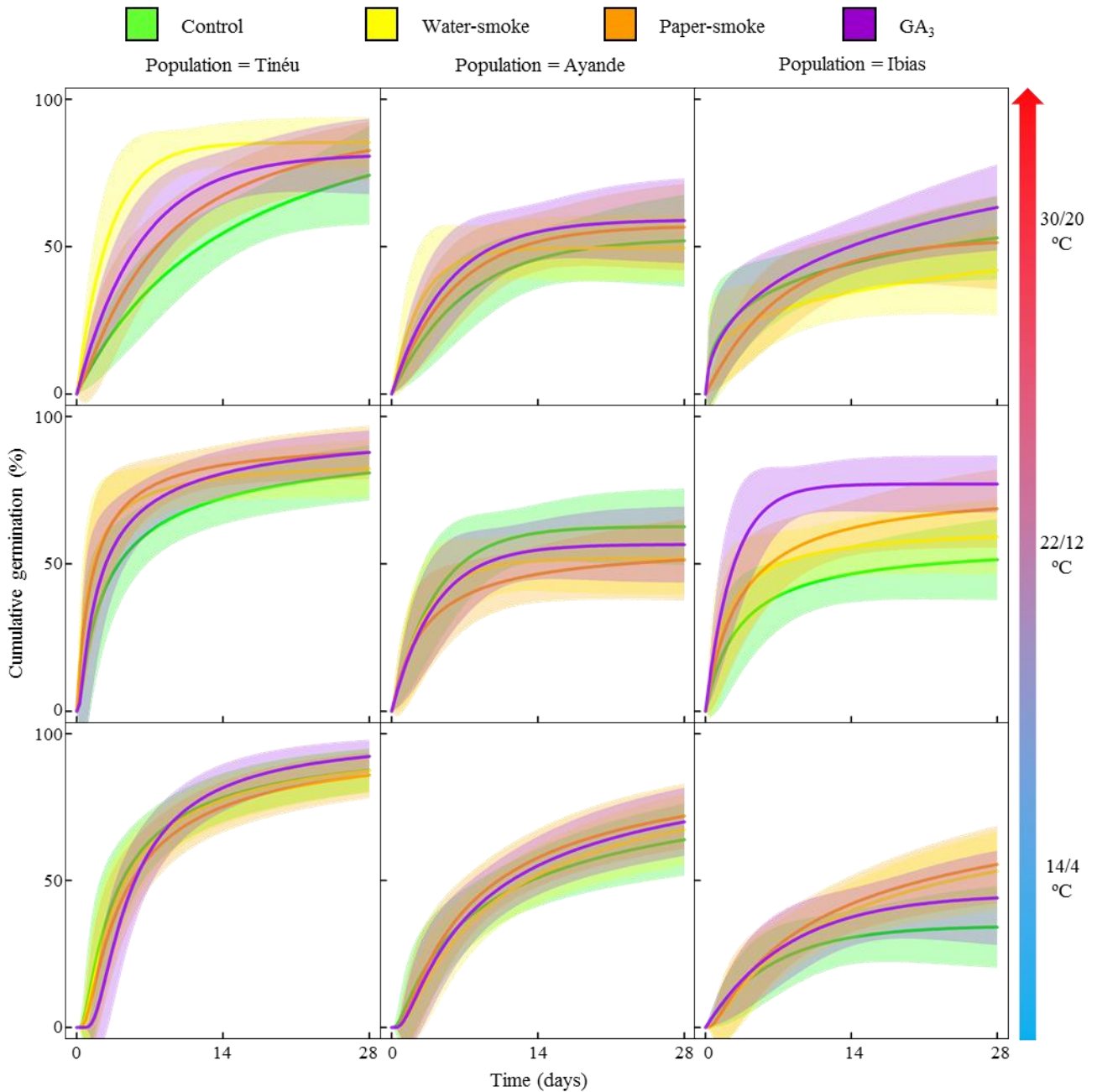
Figure 2 Germination progress of fresh Helianthemum tinetense seeds collected in three populations. Seeds were germinated at three temperature regimes, and pre-treated with distilled water (control), plant-derived smoke (water-smoke and paper-smoke) or GA3. Darker curves are growth functions fitted to data using the package 'drc'. In each treatment, a best fit function was selected between the log-logistic, asymptotic regression, Weibull and Michaelis-Menten. Lighter areas are the 95 % confidence interval of each curve. Colour online only.