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Full Length Research Paper

Optimization of epidemiological conditions to enhance the mycoherbicidal efficacy of *Alternaria alternata* against *Chenopodium album*

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A new foliage disease was found on *Chenopodium album* L. (family chenopodiaceae) in Punjab, Pakistan. *Alternaria alternata* (Fr.) Keissler was identified and confirmed as the causal agent. The present study aimed to optimize environmental condition that could enhance mycoherbicidal potential of *A. alternata* against *C. album*. In growth room trials, the effects of various inoculum concentrations of *A. alternata* (10^5, 10^7 and 10^9 conidia ml^{-1}) on disease development was studied at different growth stages of the host plant (5 - 10, 10 - 15 and 20 - 25 leaf/flowering stage) at various dew period (100% humidity for 12, 18 and 24 h) and temperature (20, 25 and 30°C) regimes. To enhance the mycoherbicidal potential of the pathogens, different formulations; 1 and 2% gelatin, 1 and 2% carboxymethylcellulose (CMC), 1:1 gelatin and CMC, 10 and 20% canola oil emulsion were used. The pathogenicity of *A. alternata* increased with increasing spore concentration and length of dew period. A spore concentration of 10^9 conidia ml^{-1} in 20% canola oil emulsion with 24 h dew period and at temperature 25 and 30°C caused 100% mortality of *C. album* plants at 5 - 10 and 10 - 15 leaf stages and resulted in maximum reduction in biomass of the target weed. The present study concludes that under a certain set of conditions, *A. alternata* can completely control *C. album*.

Key words: *Alternaria alternata*, *Chenopodium album*, conidial concentration, dew period, formulations, mycoherbicide, plant growth stage, temperature.

INTRODUCTION

Lambsquarters (*Chenopodium album* L.) is an annual weed of cultivated ground, especially on rich soils and old manure heaps (Grieve, 1984). It is often one of the first weeds to appear on newly cultivated soils (Stuart, 1979). It is considered to be a very serious weed of many crops in the world (Randall, 2003). In Pakistan, *C. album* is the most prevailing weed in the wheat fields (Siddiqui and Bajwa, 2001) and significantly reduced grain yield up to 65% through competition (Siddiqui, 2005).

Several management strategies are available to control *C. album* like manual, mechanical cultural methods and chemical herbicides. Each plant can produce over 500,000 seeds, which remain viable in the soil for up to 40 years (Royer and Dickinson, 1999). Due to the extensive production of the seeds, manual and mechanical methods are not applicable with the increasing cost of hand labour. Cultural practices are not always helpful in controlling the weed due to high adaptability potential in different conditions. Due to more labor costs and uncertainty of effects caused by mechanical methods, chemical control strategies were introduced. However, the hazards which chemicals have created in nature are more than the benefits of yield they have provided. Both health and environmental problems are associated with the use of herbicides (Soares and Porto, 2009). Furthermore, herbicide resistant weeds have become common (Llewellyn et al., 2009). Same is the case with *C. album* as it is strongly resistant to many common herbicides such as triazine (Curran, 1999). These factors have opened the way for use of plant pathogens as biological control agents of weeds. There are several reports that support the use of mycoherbicide as useful biocontrol agents tool

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in agriculture (Ellison and Barreto, 2004; Cipriani et al., 2009; Sands and Pilgeram, 2009).

An endemic pathogen might be rendered completely destructive to its weed host by applying a massive dose of inoculum at a particular vulnerable stage of weed growth (Daniel et al., 1973). A period of high humidity or leaf wetness is probably the most important environmental factor affecting the severity of disease caused by *Alternaria* spp. and other fungal pathogens (Hong et al., 1996). Temperature also plays a crucial role in the spread of disease and in general, a vast majority of fungi are unable to grow at temperatures above 35°C and below 10°C. Optimum temperature for most fungi lies between 24 and 30°C (Madan and Thind, 1998). An appropriate formulation of infective propagules, which reduces the dew requirement, would greatly improve the potential of a pathogen as a mycoherbicide (Siddiqui et al., 2009a). Recently, we reported on a new blight disease on *C. album* caused by *Alternaria alternata* which causes 60% mortality of the host plant (Siddiqui et al., 2009b). The objective of present study was to optimize the conditions for the mass destruction of *C. album* by *A. alternata.*

MATERIALS AND METHODS

Inoculum production

A. alternata was isolated from blighted areas of *C. album*. Inoculum of *A. alternata* was produced on potato dextrose agar (PDA) medium. The antibiotic streptomycin was added to prevent bacterial contamination of cultures. The media was sterilized by autoclaving at 121°C for 30 min. Twenty (20) milliliter aliquots of sterilized medium were poured in sterilized Petri plates of 9 cm diameter and allowed to solidify. A 5 mm diameter disc from 8 days old stock cultures of *A. alternata* was transferred in the centre of each Petri plate and incubated at 25 ± 1°C in a growth incubator (Company: REVCO, Model:BOD30). Conidia were collected from 8 days old cultures by scraping the agar surface with a rubber spatula. The conidial suspension was then passed through a single layer of cheesecloth to separate the conidia from mycelial debris. Conidial concentration was quantified and adjusted to 10^5, 10^7 and 10^9 conidia ml^{-1} with the aid of a hemacytometer. These conidial concentrations were selected to cover a wider spectrum of the conidial densities of *A. alternata* previously employed by other investigators in similar studies (Masangkay et al., 1999; Ghorbani et al., 2000).

Preparation of formulations

Materials used as carriers or amendments in formulations can influence the efficacy of biocontrol agents. Hence three types of materials and one combination were studied for their effects on disease development. The materials included gelatin (1 and 2% w/v), carboxymethylcellulose (CMC) (1 and 2% w/v), and canola oil emulsion (10 and 20% v/v) amended with 1 - 2 drops of mild detergent. The combination consisted of 1:1 ratio of gelatin and CMC. These materials were added to the conidial suspensions of 10^5, 10^7 and 10^9 conidia ml^{-1}.

Experimental design

A $8 \times 3 \times 3 \times 3 \times 3$ factorial experiment with 8 formulations, 3 conidial concentrations,3 growth stages, 3 temperature levels and 3 humidity levels was designed in a completely randomized manner. The formulations were sprayed on pot-grown (7 cm diameter pots) *C. album* plants at 5 - 10, 10 - 15 and 20 - 25 leaf stage (flowering stage) with a hand-operated pump sprayer until the excess fluid dripped off the foliage. Suspensions of conidia in sterilized tap water were used as positive control. For negative controls, solutions of deterrent formulations without conidia were used. The pots were transferred to a growth chamber (Model No. JS PC 420 C JS Research INC) with 12, 18 and 24 h dew period (100% humidity) at temperatures 20, 25 and 30°C for 12 h photoperiod. Each treatment was replicated three times. Data regarding the dry weight losses of shoots of target weed due to pathogen in various treatments was recorded. Since biocontrol agent generally weak the weed plants and reduce their growth, only the data regarding the shoot dry weight losses was recorded to assess the effect of biocontrol agent on weed growth.

Statistical analysis

All data were subjected to analysis of variance (ANOVA) followed by mean separation by LSD.

RESULTS AND DISCUSSION

Analysis of variance shows that the effect of temperature, humidity, conidial concentrations and formulations as well as their various interactions was significant ($P \leq 0.001$) for weed biomass as affected by *A. alternata.* The effect of growth stage of weed was insignificant. However, the interactive effect of growth stages with other variables was significant for shoot dry biomass production (Table 1).

Data regarding the effect of different mycoherbicidal formulations on shoot dry biomass of *C. album* at three stage stages, three conidial concentrations, three temperature regimes and three humidity levels is illustrated in Figures 1 - 3. In general, the effect of different variables on disease severity and weed biomass reduction was similar at different growth stages. However, plants were comparatively less susceptible to disease at flowering stage (20 – 25 leaf stage). At 20°C, the effect of all the mycoherbicidal formulations on shoot dry biomass of *C. album* was insignificant. The effect of different humidity levels was also insignificant at this temperature. At 25 and 30°C, both 10 and 20% canola oil emulsion mycoherbicidal formulations markedly enhanced the disease severity; consequently shoot dry biomass of the target weed species was reduced. Adverse effect of the two formulations on shoot biomass was increased with an increase in conidial concentration and humidity duration. Maximum mycoherbicidal potential of *A. alternata* was recorded at 24 h humidity level and 10^9 conidia ml^{-1}. The effect of 20% canola oil emulsion was more pronounced as compared to 10% canola oil emulsion. The effect of other mycoherbicidal formulation on shoot dry biomass reduction was generally insignificant.

All the growth stages of weed when exposed to mycoherbicidal formulations responded well in terms of

Table 1. Analysis of variance (mean squares) for dry weight percentage losses due to blight disease in *C. album*.

Source of variation	df	SS	MS	F values
Growth stage (S)	2	11	5.7	1.2 ^{ns}
Temperature (T)	2	912	456	98*
Humidity (H)	2	346	173	37*
Conidial concentration (C)	2	155	78	17*
Formulations (F)	7	3912	559	120*
S×T	4	345	86	19*
S×H	4	173	43	9.3*
S×C	4	396	99	21*
S×F	14	172	12.3	2.6*
T×H	4	132	33	7*
T×C	4	203	51	11*
T×F	14	1574	112	24*
H×C	4	332	83	18*
H×F	14	845	60	13*
C×F	14	172	12.3	2.7*
S×T×H	8	392	49	11*
S×T×C	8	410	51	11*
S×T×F	28	956	34	7.3*
S×H×C	8	356	45	9.6*
S×H×F	28	1350	48	10*
S×C×F	28	1211	43	9.3*
T×H×C	8	391	49	10.5*
T×H×F	28	599	21	4.6*
T×C×F	28	577	21	4.4*
H×C×F	28	1594	57	12*
S×T×H×C	16	436	27	5.9*
S×T×H×F	56	1587	28	6*
S×T×C×F	56	1392	25	5.3*
S×H×C×F	56	1688	30	6.5*
T×H×C×F	56	1902	34	7.3*
S×T×H×C×F	112	2651	24	5.1*
Error	1296	6037	4.7	
Total	1943	33215		

*Significant at P≤0.001; ns, non-significant.

disease development. However, in case of younger growth stages (5 – 10 and 10 – 15 leaf stages) in *C. album*, disease spread was maximum at 25 – 30°C with 100% humidity for 24 h and conidial concentration of 10^9 conidia ml^{-1} of *A. alternata* in 20% canola oil emulsion. The mature, flowering stage was not so susceptible to the pathogen. The high susceptibility of the younger seedlings to bioherbicidal agents has also been reported in other studies (Leger et al., 2001; Peng et al., 2004). However, in some contrasting reports, older plants have been shown to be more susceptible to their respective pathogens and lower susceptibility of the younger plants was attributed to the ability of the actively growing host tissue to partially outgrow the disease (Makowski, 1993). These controversial findings probably can be attributed to species-specific responses and specific host pathogen interactions.

The conidial concentration of 10^5 and 10^7 conidia ml^{-1} could not produce the desired levels of disease. The application of conidial concentration of 10^9 conidia ml^{-1} provided the best results in causing severe disease on target weed. This led to the conclusion that higher inoculum density of potential mycoherbicides is required to get desired level of plant mortality. These observations are in conformity with findings of other workers (Kadir et al., 2000; Ghorbani et al., 2002; Peng et al., 2004).

The results obtained in the present study indicated that humidity period of 24 h was most effective in causing

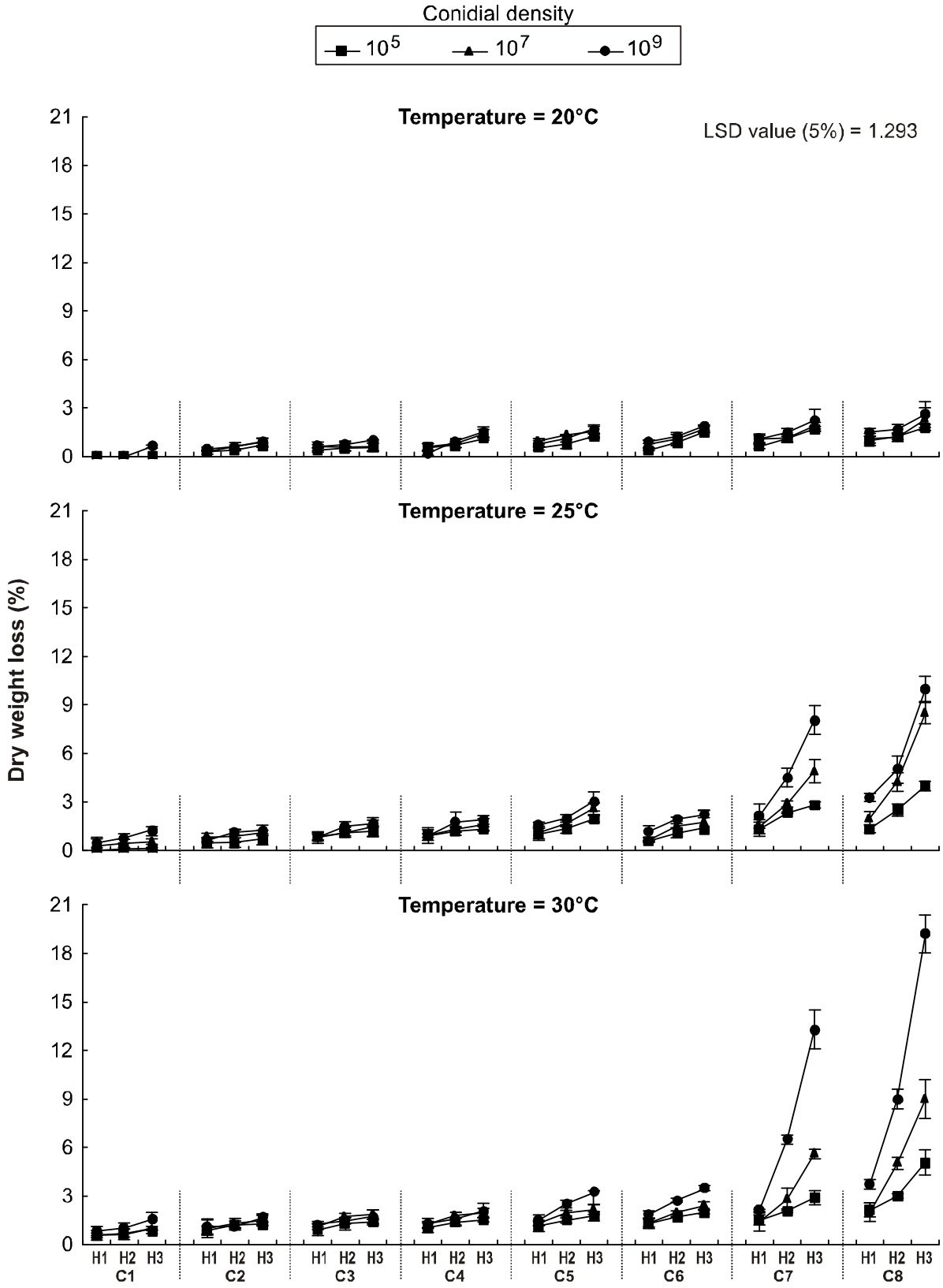

Figure 1. Effect of different conidial concentration of *A. alternata* at different humidity and temperature regimes on dry weight losses of *C. album* at 5 – 10 leaf stage. Vertical bars show standard errors of means of three replicates. C1 = Water; C2 = 1% Gelatin; C3 = 2% gelatin; C4 = 1% CMC; C5 = 2% CMC; C6 = 1:1 gelatin: CMC; C7 = 10% oil emulsion; C8 = 20% oil emulsion; H1 = 12 h humidity, H2 = 18 h humidity, H3 = 24h humidity.

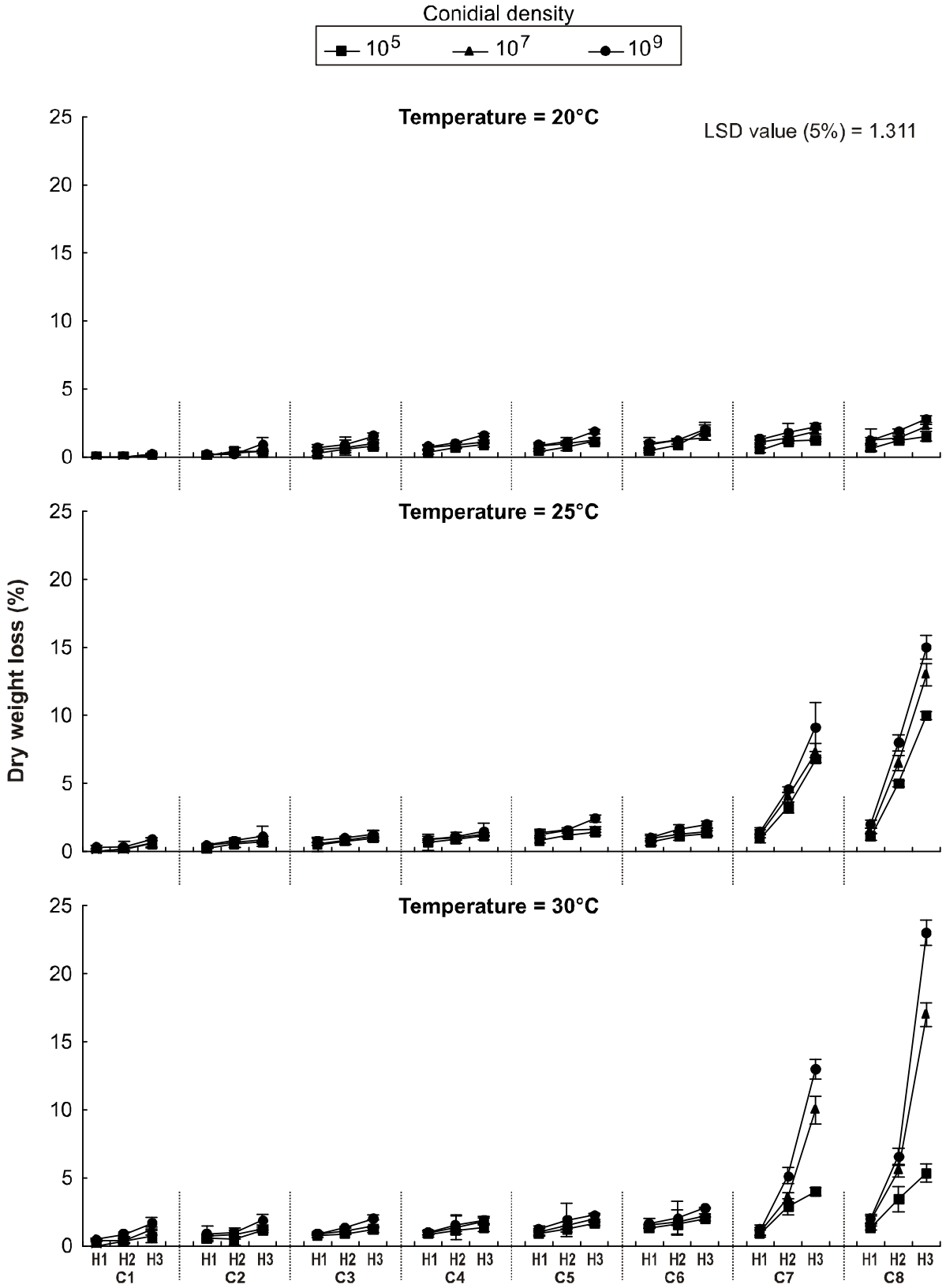

Figure 2. Effect of different conidial concentration of *A. alternata* at different humidity and temperature regimes on dry weight losses of *C. album* at 10 – 15 leaf stage. Vertical bars show standard errors of means of three replicates. C1 = Water; C2 = 1% gelatin; C3 = 2% gelatin; C4 = 1% CMC; C5 = 2% CMC; C6 = 1:1 gelatin: CMC; C7 = 10% oil emulsion; C8 = 20% oil emulsion; H1 = 12 h humidity, H2 = 18 h humidity, H3 = 24 h humidity.

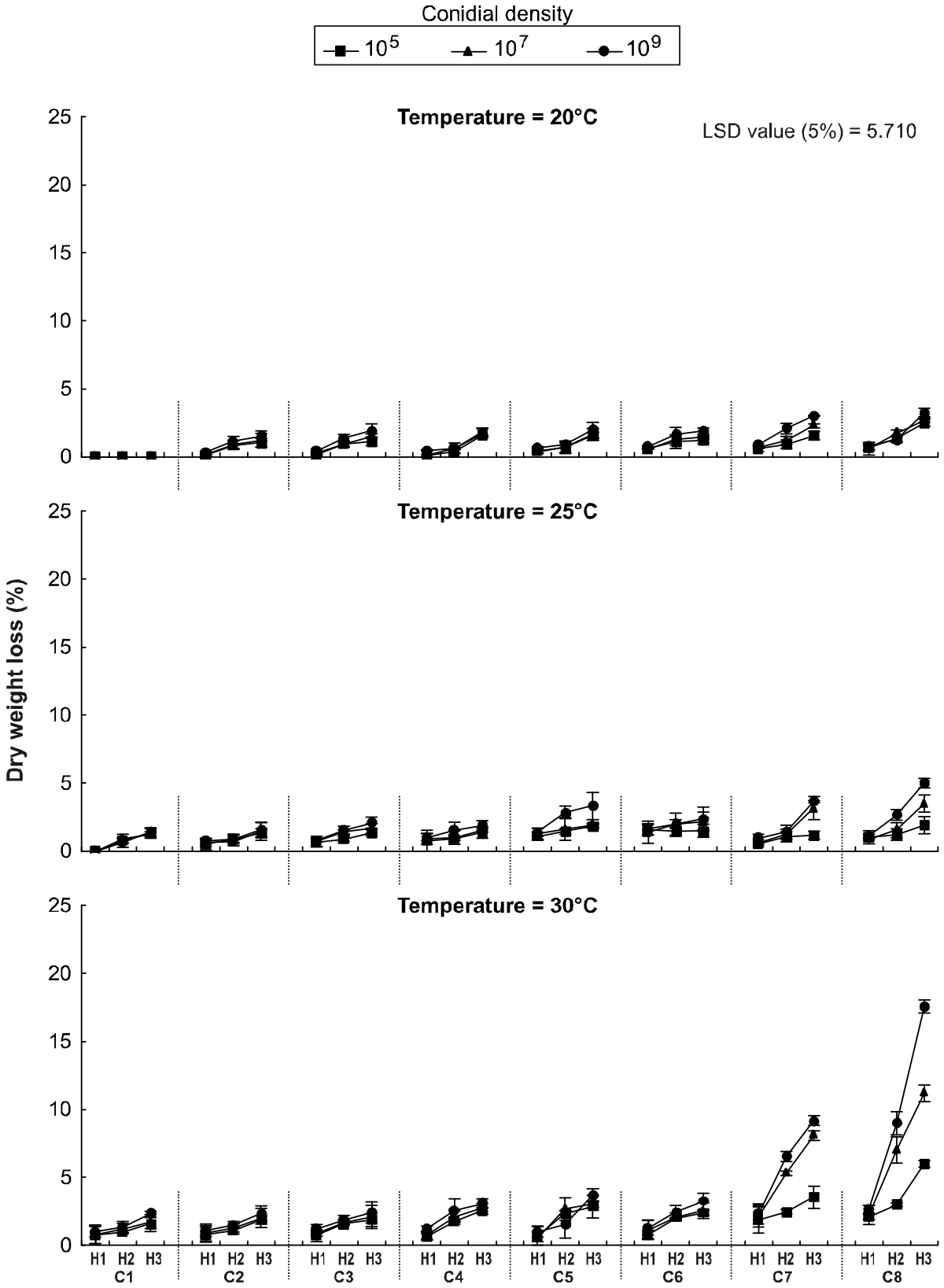

Figure 3. Effect of different conidial concentration of *A. alternata* at different humidity and temperature regimes on dry weight losses of *C. album* at 20 – 25 leaf stage. Vertical bars show standard errors of means of three replicates. C1 = Water; C2 = 1% gelatin; C3 = 2% gelatin; C4 = 1% CMC; C5 = 2% CMC; C6 = 1:1 gelatin: CMC; C7 = 10% oil emulsion; C8 = 20% oil emulsion; H1 = 12 h humidity, H2 = 18 h humidity, H3 = 24 h humidity.

disease spread and consequent decline in dry weight of diseased plants as compared to healthy ones. The need for high humidity duration was realized because the foliar pathogens require free moisture to infect plants, which directly affect the disease development. These results are in line with the findings of Ghorbani et al. (2000) and Khan and Hsiang (2003). A requirement of more than 12 h of dew period for severe infection has also been reported for several potential mycoherbicides (Makowski, 1993; Kadir et al., 2000). However, there is a lot of controversy with reference to involvement of humidity periods. In some cases, long dew period has been shown to be responsible for the poor performance of many fungal bioherbicides (Watson and Wymore, 1990; Charudattan, 1991).

Maximum weed biomass reduction due to disease was recorded at 25 and 30°C. Temperature plays a crucial role in the spread of disease and in general, a vast majority of fungi are unable to grow at temperatures above 35°C and below 10°C. Ghorbani et al. (2000) showed that optimum temperature for disease development and consequent dry weight reduction by *A. alternata* on *Amaranthus retroflexus* plants was 20 to 30°C. Optimum temperature for most fungi lies between 24 and 30°C (Madan and Thind, 1998). Post inoculation temperature is likely to have significant effects on both penetration and subsequent mycelial growth (Walker, 1981).

Water suspension was found to be the least effective as compared to the rest of the formulations in disease spread and reduced the shoot dry biomass only at the optimum temperature of 25 and 30°C and 100% humidity for a period of 24 h. This is because water lacks the effective sticking quality and has the least ability to be absorbed at the leaf surface. Formulations like CMC and gelatin produced better results in terms of disease expression and weight loss. Materials capable of forming aqueous gels when added to spore suspensions; these helped in sticking the spores to the leaf surface, while providing a hydrophilic matrix in which the spores may germinate more readily in the absence of an adequate dew period (Shabana et al., 1997). Overall disease spread was greater in CMC than gelalin and thus greater shoot dry weight reduction of target weed was obtained at the optimum conditions of temperature and humidity. This is due to the presence of cellulose in CMC, which made it better carbon source for the fungus (*A. alternata*) than gelatin, which only acts as sticking agent. With the activity of enzymes, cellulose is broken down into trisaccharides etc. which provide growth media to the fungi. In case of *A. alternata,* best growth results have been obtained on trisaccharides such as rafinose (Madan and Thind, 1998). Gelatin and CMC, used together in 1:1 ratio declined disease incidence with consequent reduction in shoot biomass. This might be due to a complex interaction of gelatin and CMC, causing depression in transpiration rate by closing of stomata and thus affecting the mechanism of photosynthesis with ultimate decline in weight of the plants. The maximum spread of disease was supported by 20% canola oil emulsion followed by 10% canola oil emulsion. Emulsions can increase the survival and germination rate of spores applied to leaves (Amsellem et al., 1990). The oil emulsions with different amendments have been found to be effective in *A. alternata* mycoherbicidal treatments (Ghorbani et al., 2000; Auld et al., 2003). Reports have indicated that formulation in the form of canola oil was better than other oil emulsions (Auld, 1993). The use of oil emulsion has certain advantage over CMC and gelatin because it forms a much thinner layer on the leaf surface as compared to the gel forming materials, which not only allows for the better growth of conidia but also does not prevent the exchange of gases in the leaf.

Conclusion

The present study concludes that *A. alternata* can control *C. album* effectively when 10^9 conidia ml^{-1} in 20% canola oil emulsion is sprayed on the weed at 5 – 15 leaf stages under 100% relative humidity for 24 h. There are two major limitations for using *A. alternata* as a mycoherbicide against *C. album*. Firstly, the high spore density which can increase the cost application when used as a commercial product and secondly, the long dew period (24 h) which cannot be achieved in many climatic zones of the world and even in the same country. Further research is therefore required to improve the efficacy of this pathogen by using more effective strains of *A. alternata* which can show their potential with low spore density and short dew period.

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