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# POTENTIAL OF PLANT GROWTH REGULATOR AND CHLORMEQUAT CHLORIDE ON ALFALFA SEED COMPONENTS

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**Abstract**

The use of plant growth regulators (PGRs) has opened new prospects for increased seed production in grasses and legumes, but little information is available on the effects of PGRs combination with chlormequat chloride (CCC) on alfalfa (*Medicago sativa* L.) seed yield components. This study was conducted to evaluate the effects of applying chlormequat chloride in combination with three PGRs (Naphthylacetic acid (NAA), gibberellic acid 3 (GA), and brassinolide (BR)) on seed yield, aboveground biomass, plant height, lodging, yield components. CCC was applied annually at the stooling stage while three PGRs were applied twice each year at the stages of flower bud formation and peak flowering. Results provides evidence that: (i) each PGR consistently increased seed yields, and the numbers of seeds per stem compared to untreated plants; (ii) CCC treatment reduced plant height and lodging, but also significantly decreased seed yield and did not affect aboveground biomass. (iii) effectiveness of CCC application depends on climatic conditions, especially in North-east China. (iiii) the optimum combination of CCC with a PGR to increase alfalfa seed production was failed to identify. (iiiii) no interactions between PGRs and CCC on seed yield were observed and neither the PGRs nor the CCC. But alfalfa seed yield could be improved by combining a PGR such as NAA. Our results suggest that these PGRs could be used in alfalfa breeding to increase seed yield while maintaining high seed quality.

**Key words:** Plant growth regulators, Chlormequat chloride, Alfalfa (*Medicago sativa* L.), Seed yield.

## Introduction

Alfalfa is one of the most grown perennial forage crops worldwide, due to its great yield potential, high nutritive value, and wide adaptation. With high biomass yield and nutrition, alfalfa is grown mainly for forage production and seed yield is considered to be of secondary importance (Iannucci *et al*., 2002). Because of seed yield is a complex trait (Dragovoz *et al*., 2002) , the seed yield is quite variable ranging from 60 to 1, 000 kg $ha^{-1}$ (Iannucci *et al*., 2002), and another problem associated with the seed production is the poor seed quality. However, researches have shown that alfalfa seed yield improvement achieved through breeding has been limited (Falcinelli, 2000), for example that different alfalfa growing regions have different constrains for realization of seed yield potential. Dry and hot regions have problems with drought resistance, acid soil regions–tolerance to low pH and aluminum, wet and cool regions – lodging and diseases and so far. (Iannucci *et al*., 2002; Bolanos-Aguilar *et al*., 2002). Furthermore, much work has mainly focused on improving forage quantity and quality rather than alfalfa seed yield. Therefore, it is of importance to improve seed yield to employ agronomic practices.

Plant growth regulators (PGRs) have be evaluated with regard to alfalfa seed yield and have opened new prospects for increased seed production in grasses and legumes such as the pea (*Pisum sativum* L.) and the soybean [*Glycine max* (L.) Merr.] (Lorenzetti, 1993). In these legume plants, available energy is partitioned to seed production, such that vegetative reserves are mobilized to fill the seeds. Contrarily, the balance between vegetative and reproductive phases in alfalfa seed yield is often indeterminate. In fact, the seed production rate of alfalfa is very low and its vegetative biomass is greater. As the overall amount of assimilation can be considered nonlimiting, the main factor responsible for low alfalfa seed yield could be the distribution of assimilates, and several factors negatively influencing distribution of assimilates could be concerned (Genter *et al*., 1997). Lorenzetti (1993) reported that the use of synthetic growth regulators makes it possible to obtain seed yield in grasses such as wheat (*Triticum aestivum* L.), rice (*Oryza sativa* L.), and barley (*Hordeum vulgare* L.). Furthermore, one may reasonably anticipate similar effects in legumes such as alfalfa. Rijckaert (1991) reported that the use of a PGR could increased seed yield of white clover and Veverka *et al*. (1993) found a marked rise in the number of alfalfa pods other than slight growth inhibition. Although a combination of two or more PGRs has been applied to increase the yields of many crops, such as rice, wheat, and cotton (*Gossypium hirsutum* L.), only application of a single PGR have been investigated in alfalfa. There are some reported by Skalaska, (1994) and Wang, (2005) that chemicals test include TIBA (2, 3, 5 – triiodobenzoic acid), daminozide [butanediotic acid mono (2, 2 – dimethylhydrazide)], CCC (chlormequat chloride), and paclobutrazol. As far as our known, CCC can inhibits gibberellin biosynthesis and is widely used in soybean and wheat farming to decrease plant height and prevent lodging (Zhang *et al*., 2009). Therefore, application of an additional PGR with CCC may anticipate beneficial effects in improving the reproductive development of alfalfa. Although the combined application of CCC with PGRs have been previously reported for alfalfa seed production (Zhang *et al*., 2009), combination PGR treatments associated with the seed production remain a concerned theme.

In this field study, the combined application of CCC with three PGRs were applied from 2008 to 2011. The purpose of this study was to the influence of the combined application of CCC with PGRs on the seed production of alfalfa widely grown in the Songnen Plain of the northeast China. Our objectives in this study were (i) to compare the individual effects of the PGRs on seeds yield and components; and (ii) to determine the combined effects of CCC and each of the three PGRs on alfalfa seed yield. (iii) to determine which combination method had higher seeds yield.

## Materials and Methods

**Region and site description**: The field experiment was carried out at the Frigid Forage Research Station located at Lanxi county (Fig. 1), run by Heilongjiang Academy of Agricultural Sciences (HASS). The station has an altitude of 160 m, longitude of 125°58', 46°32' N in Northeast China (Chen *et al*., 2014). All seeds were sown on an experimental field which is situated in the northwest margin area of the Songnen Plain in 2008. The climate is classifed as a typical chillness semiwetness monsoon environment. Based on data from 1988 through 2008, the total yearly sunshine duration is 2713 hours and the no frost period is 130 days. The annual mean air temperature is 5.3°C with a maximum temperature of 31.2°C (July) and a minimum temperature of -25.2°C (January). The annual mean accumulated heat units (above 10°C) is 2,760°C. The annual mean precipitation is 469.7 mm, of which about 75% falls from June to August and the average annual free water evaporation is about 950 mm. In this study, the weather conditions (monthly maximum and minimum temperatures and total rainfall for 2008 and 2011) were recorded (Fig. 2). The soil is dark loam (mostly Chernozem, FAO Taxonomy) with high melanic humus. The experimental area had an average soil pH of 8.12, an average soil organic matter content of 6.04%, total N content of 0.34%; the contents of $NO^{3-}$ - N was 4.35%, the contents of $NH^{4+}$ - N was 6.81% and available P was 22.35 ppm (Olsen method). The experiment was seeded on 1 May 2008 and crops grew for two years until 2011.

The alfalfa seed of variety 'Zhaodong' was provided by HASS. This variety was chosen due to its high adaptability and existing widespread use in the northeast region of China. The experimental plots were established and seeded on 1 May of 2008. Each plot is 3 meter long and 2 meter wide with inter-row spacing of 15cm. Seeding rate was 7.5 kg $ha^{-1}$ and seeds were drilled uniformly. Seeds were inoculated with a commercial inoculant of *Sinorhizobium* (Chen *et al*., 2014). After seeding, the plot surface was pressed using a corrugated roller. Open perimeter area outside of the experiment was reserved to protect the experimental rows from interferential damage. No fertilizer or irrigation was applied during the experimental periods. Plots were 2m×3m and hand-weeded during the growing period whenever necessary for proper weed control. In addition, the experiments were carried out for three consecutive years. Alfalfa leaf – cutting bee (*Megachile rotundata* F) is the one of honeybees, which primarily provided (3, 5000 heads $ha^{-1}$) pollination during the seed production years.

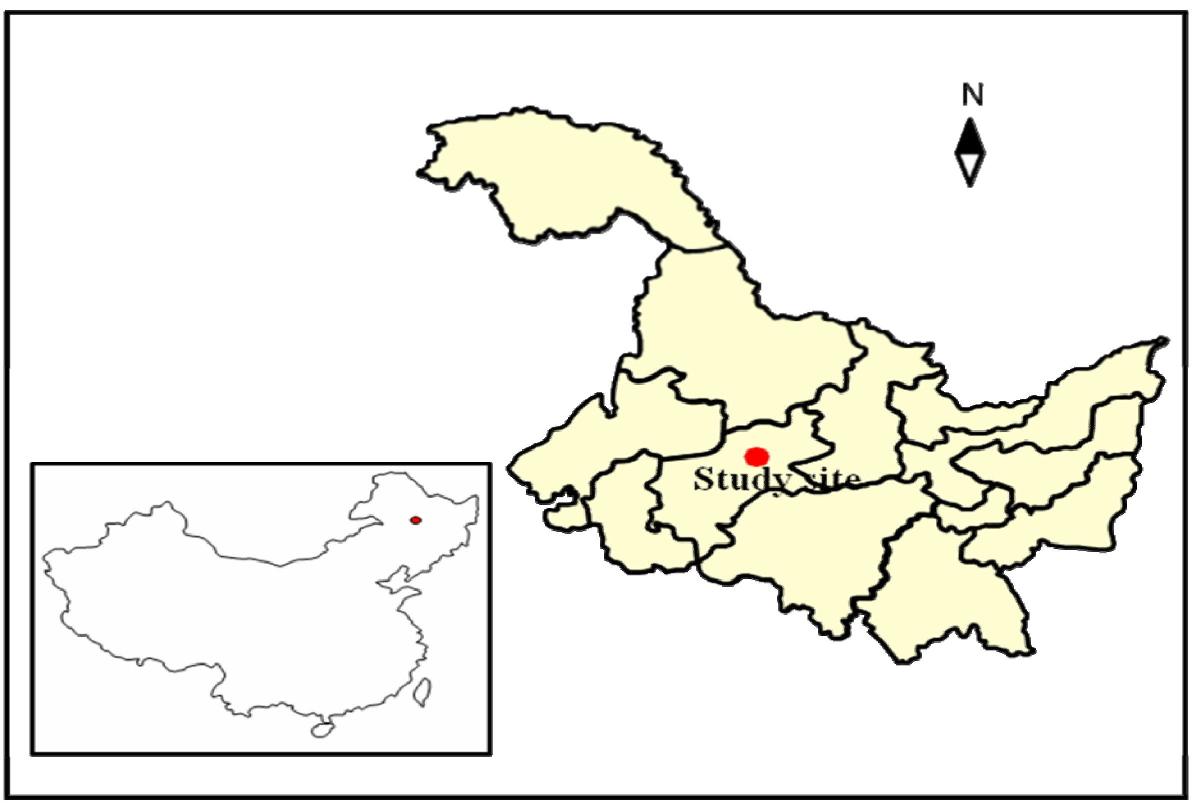


Fig. 1. The location of Heilongjiang province in China and the location of the study site in Lanxi county.

**Table 1. Application dates of PGRs and CCC in 009, 2010 and 2011year.**

| | 2009-year | 2010-year | 2011-year |
|---|---|---|---|
| Stooling data | 1 June | 1 June | 2 June |
| Bud data | 12 June | 13 June | 13 June |
| Flowering data | 18 June | 19 June | 19 June |

**Experimental design:** The experimental design was a split – plot with four replications. Two CCC treatments were applied in the main plots and the three PGRs were applied in the subplots. The main treatments consisted of CCC and CK. The CCC was sprayed of 5 kg a.i. $ha^{-1}$ and the CK consisted of clean water sprayed at 75 L $ha^{-1}$ to cover all plant surface. The side treatments consisted of the CK (water sprayed at 75 L $ha^{-1}$) and the three PGRs used reportedly improve chlorophyll concentrations, as well as the photochemical efficiency of soybean and wheat. In this study, the PGRs includes (i) naphthylacetic acid application of 20 g a.i. $ha^{-1}$ (NAA), (ii) gibberellic acid 3 application of 20 g a.i. $ha^{-1}$ (GA), and (iii) brassinolide application of 6 mg a.i. $ha^{-1}$ (BR).

The main treatments were randomly assigned to the main plots within replicates, and the three PGRs and CK were randomly assigned within each subplots. Each replicate contained 8 treatment combinations and each subplot received the same treatment combination each year. Each individual subplot is 3 meter long and 2 meter wide with inter – row spacing of 15cm. The CCC was applied annually at the shooting stage to control the alfalfa height. The PGRs were applied twice each year at the stages of squaring stage and flowering stage. The table 1 is about application dates of PGRs and CCC in 2009, 2010, and 2011. Since no specific levels have been indicated for alfalfa seed production, the concentrations of GA and BR were based on the manufacturer's recommended rates for soybean and wheat production. The concentrations of CCC and NAA were selected based on previous research reported by Wang *et al*. (2005) and Zhang *et al*. (2009). During the second year of the alfalfa stand, application of the chemicals were initiated in 2009. All chemicals with a sprayer that delivered 75 L $ha^{-1}$ at 290 kPa were applied before 09: 00 h or after 15: 00 h to avoid the effects of sunshine.

**Sampling and analytical methods:** Plots were harvested when 75% of the pods turned blackish brown and when the seed moisture content reached approximately 17%. Seed yields were determined from a random 3.0 $m^2$ sample from each plot harvested by hand. The seed samples were dried, threshed, cleaned, weighed, and stored in paper bags before laboratory analysis. Seed yield was calculated at a standard seed moisture content of 13% (kg $ha^{-1}$). Another 3.0 $m^2$ area per plot was sampled to determine the aboveground biomass (AGB). Fresh weights were determined, and subsamples (30 stems) were then dried at 60°C to obtain a constant dry weight. The AGB was calculated on a dry – weight basis (Li *et al*., 2013).

The variables of seed yield components included stems per $m^2$ (number of shoots determined by 1 $m^2$ samples randomly collected from each plot before seed harvest), racemes per stem (random samples of 30 stems each plot before seed harvest), pods per raceme (random samples of 60 racemes each plot before seed harvest), seeds per pod (random samples of 60 pods each plot before seed harvest), and 1000 – seed weight (g). The 1000 – seed weight was determined using three random samples of clean seeds from each plot that had previously been dried at 80°C to constant moisture content. An integrated component, seeds per stem, was calculated from racemes per stem, pods per raceme, and seeds per pod.

Plant height was determined by selected randomly from each plot before harvest. Lodging was evaluated visually using a 1 to 5 scale (1 indicating no lodging and 5 indicating 100% lodging) for each plot during the flowering period. The laboratory germination rate was performed on three replicates of 100 seeds per sample for each plot. Normal seedlings of at least 1.5 cm length were counted and removed each day. Percentages of normal seedlings and hard seeds were calculated at the end of the test, consistent with ISTA guidelines (Anon., 1999).

**Statistical analysis:** The experiment was conducted for three consecutive years (2009, 2010, and 2011) in one location. Years were first treated as a random factor to analyze the effects of years, two treatments and their interactions using a general linear model (GLM) procedure with two-way ANOVA and a posteriori Tukey – Kramer LSD test ($p$<0.05), which were used to examine the main and interactive effects of combination treatments on stems per $m^2$, racemes per stem, pods per raceme, seeds per pod, 1000 – seed weight of alfalfa seeds, plant height, seeds per stem, aboveground biomass, and germination rate. Due to a lack of normality, some data were square root transformed as appropriate prior to analysis. Where F – tests were significant ($p$<0.05), LSD was calculated to compare the means. All data were assessed for homogeneity of variance and normality and statistical analyses were performed using statistical computer software SPSS 19.0 (SPSS, Chicago, IL.).

## Results

**Seed yield comparisons among different years:** The experiments were carried out for three consecutive years (2009, 2010, and 2011), where average annual seed yield from different year was different and variable. In three growing seasons, and the best year for alfalfa seed production was 2010 year followed by 2009 year, while the smallest seed yield was found in 2011 year (Fig. 2).

**Seed yield comparisons among different treatments:** Across the three consecutive years (2009, 2010, and 2011year), average seed yields from different CCC treatments were significantly affected ($p$<0.05) and suggest that optimum seed yields were found in 2010 year, followed by 2009 year, while the smallest seed yield was found in 2011 year.

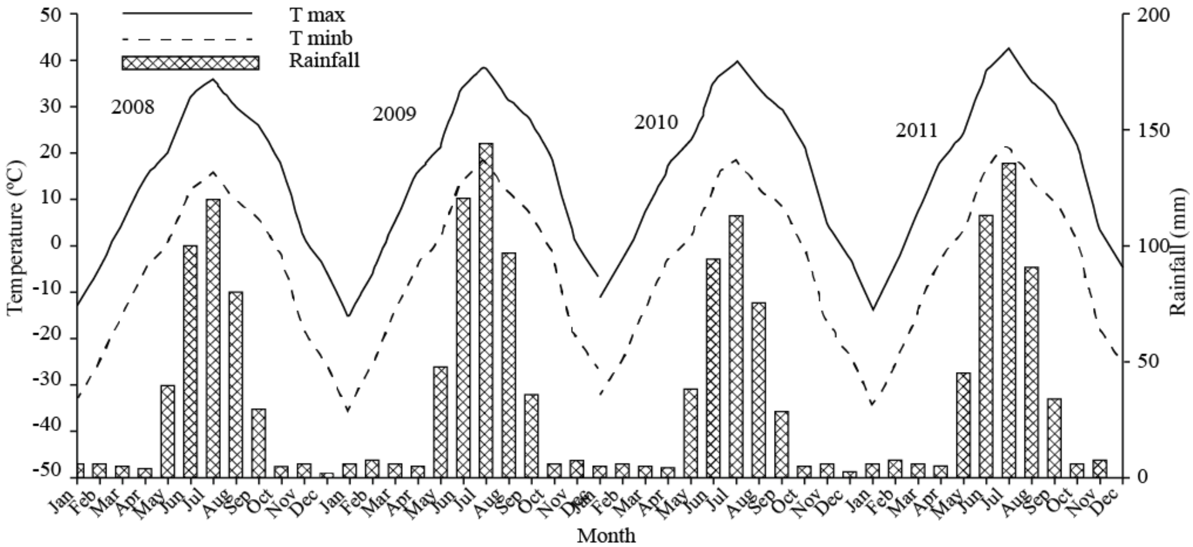


Fig. 2. Monthly maximum and minimum temperatures and total rainfall for 2008 and 2011 at Lanxi county, China.

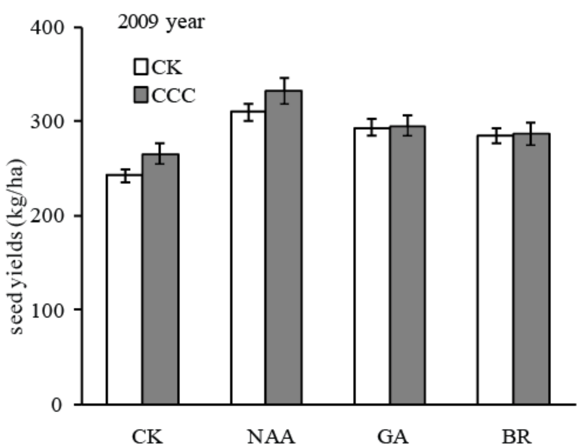


Fig. 3. Average values for seed yield under the combination of plant growth regulator and chlormequat chloride treatments in 2009 year. T bars represent SEM for 6 treatment combinations.

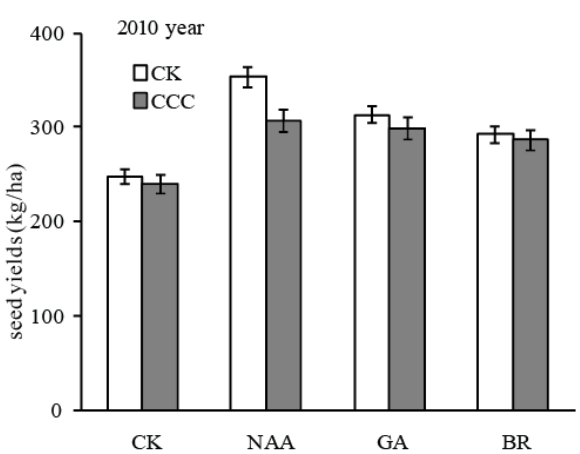


Fig. 4. Average values for seed yield under the combination of plant growth regulator and chlormequat chloride treatments in 2010 year. T bars represent SEM for 6 treatment combinations.

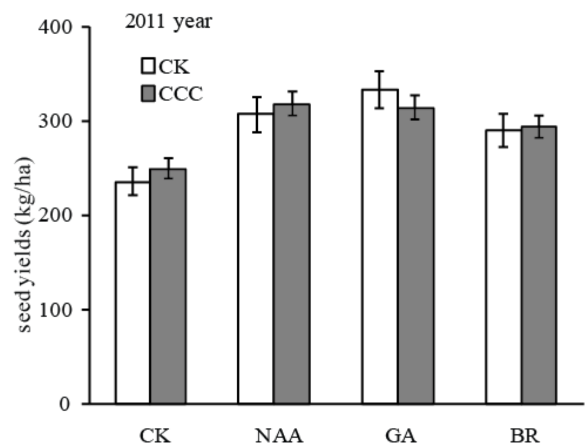


Fig. 5. Average values for seed yield under the combination of plant growth regulator and chlormequat chloride treatments in 2011 year. T bars represent SEM for 6 treatment combinations.

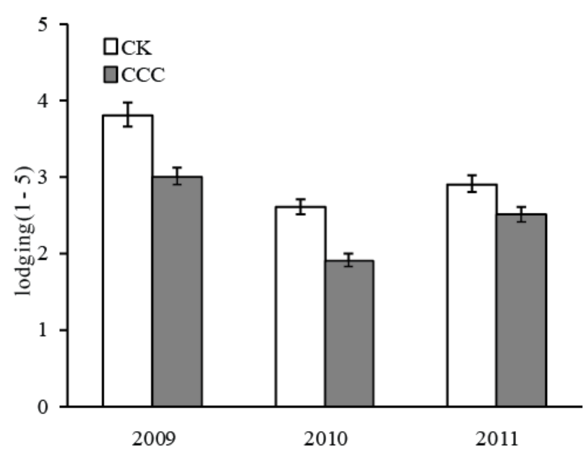


Fig. 6. Average values for plant height under CCC treatments in 2009, 2010, and 2011 year.

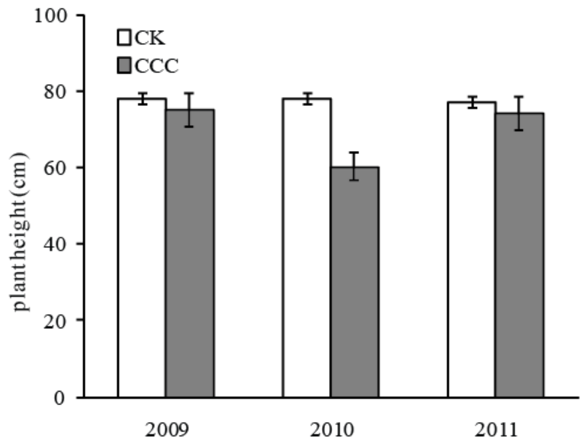


Fig. 7. Average values for lodging under CCC treatments in 2009, 2010, and 2011 year.

**Table 2. Average of seed yield (kg ha$^{-1}$) under PGRs and CCC treatments in 2009, 2010 and 2011 year.**

| | 2009-year | 2010-year | 2011-year |
|---|---|---|---|
| ***Three PGRs*** | | | |
| CK | 689 | 655 | 753 |
| NAA | 956 | 769 | 1055 |
| GA | 823 | 765 | 853 |
| BR | 727 | 586 | 823 |
| ***CCC*** (kg a.i. ha$^{-1}$) | | | |
| 0 | 820 | 682 | 1022 |
| 5 | 869 | 792 | 924 |
| ***ANOVA*** | | | |
| PGR | * | * | * |
| CCC | * | * | * |
| PGRs + CCC | NS | NS | NS |

Notes: * Significant at the 0.05 probability level. NS, Not significant.

**Table 3. Average of AGB (kg ha$^{-1}$) under PGRs and CCC treatments in 2009, 2010 and 2011 year.**

| | 2009-year | 2010-year | 2011-year |
|---|---|---|---|
| ***Three PGRs*** | | | |
| CK | 6189 | 6155 | 7513 |
| NAA | 9516 | 7619 | 9655 |
| GA | 8123 | 7265 | 8253 |
| BR | 7127 | 6886 | 8923 |
| ***CCC*** (kg a.i. ha$^{-1}$) | | | |
| 0 | 8220 | 6382 | 10122 |
| 5 | 8369 | 7592 | 9324 |
| ***ANOVA*** | | | |
| PGRs | NS | NS | NS |
| CCC | * | * | NS |
| PGRs + CCC | NS | NS | NS |

Notes: * Significant at the 0.05 probability level. NS, Not significant.

**Seed yield comparisons among different combinations:** From Figs. 3, 4 and 5, no one of treatment combinations consistently resulted in the greatest seed yields during the three consecutive years .The combinations of NAA with CCC, NAA alone, and GA with CCC resulted in maximum yields in 2009, 2010, and 2011 (Figs. 3, 4 and 5).

**Aboveground biomass comparisons among different combinations:** In this study, date from three years had no significant differences in AGB among the three PGRs and the untreated plants (Table 3).

**Plant height and lodging comparisons among different combinations:** Effects of CCC and PGR were also apparent in plant height. When averaged across PGR, CCC significantly reduced plant height (cm), with 4.1, 6.2, and 2.7% reductions over untreated plants in 2009, 2010, and 2011yaer, respectively (Fig. 6).

## Discussion

Based on the weather in July of 2009 year and 2011 year from figure 2, much more precipitation (22.4 and 20.2 mm) were comparative to the average value (18.1 mm) so that more lodging occurred in both years. So both years were detrimental to pollination and seed set, resulting in lower seed yields. This result finding is consistent with those of similar studies by Bolanos – Aguilar *et al*. (2002) and Iannucci *et al*. (2002) on alfalfa seed yield in the United States

Although CCC has been proved beneficial to pollination and seed set (Wang *et al.*, 2005; Skalaska, 1994). In this study, CCC applied at a rate of 5 kg a.i. ha$^{-1}$ significantly decreased seed yield by 1.9%. The similar result was reported by Kalmer (1991) that high concentrations of CCC on alfalfa induced some diseases. However, compared with the control, there were increases of 4.2 and 9.3% in 2009 and 2011 year, but a decrease of 13.8% in 2010 with the CCC application. During testing period, the excessive precipitation in July of 2009 and 2011 year was probably detrimental to pollination and seed set (it mean that honey bees pollinating activity was influenced) (Goldman & Dovrat, 1980; Hutmacher *et al*., 1991), and this variable responses support the findings that climate conditions greatly influence the effects of CCC as reported by Wang *et al*. (2005) and Zhang *et al*. (2009).

To the three PGRs, average seed yields in each year varied significantly (Table 2). Greatest seed yields were obtained with NAA in 2010, whereas maximum yields were obtained with GA in 2010 and with BR in 2010. Furthermore, NAA resulted in the greatest average seed yields over the 3-yr study, which seed yields was 3103.33 kg ha$^{-1}$, an over all increase of 19.38%. This finding agrees with other studies reported by Wang, *et al*. (2005), Dhaliwal and Bains, (1983) that application of NAA during anthesis may improve alfalfa seed yield in some years. Therefore, two applications of NAA at the stages of squaring and flowering were more efficient in increasing alfalfa seed yield as compared to one application during anthesis. In comparison, the report by Yadava *et al*. (1984) considered that high concentrations of NAA restrained and decreased alfalfa seed yield. Therefore, during the reproductive development of alfalfa, it may be recommendative that multiple applications of low to moderate rates of NAA rather than a one-time application at a higher rate has greater potential for improving seed yield. GA treatment increased seed yield over the control (CK) by 13.7% (2009), 37.1% (2010), and 22.5% (2011) with a mean increase over 3 yr of 23.5%. However, it was reported that applying GA during the formation of lateral shoots had little effect on alfalfa seed yield (Skalaska, 1994). This difference in results is most likely due to the applications of GA twice in this study such that this approach may have increased its activity and prolonged the duration of effectiveness. A similar trend was observed that BR treatments increased the mean 3-yr seed yields by 21.3, 21.5, and 16.6%, respectively, suggesting the potential of these PGRs to increase alfalfa seed yield. This result finding is consistent with papers published in the agronomic literature related to the effects of BR on alfalfa seed yield (Zhang *et al*., 2009). However, the application rates and timings used in this study are not necessarily optimal for alfalfa. Further research is necessary to better understand this issue.

For the same PGR combination treatments, comparisons between control and CCC were consistent with those subplots in which no PGR was used. There were no significant interactions between CCC and PGR treatments over 3 year (Table 2), suggesting that both treatments acted independently, and the combined effects can be calculated from individual results. This could partially result from a lack of CCC activity by half a month after CCC application when the PGRs were applied.

The untreated plants had a slightly greater mean AGB over 3 yr, but significantly lower mean seed yield compared with the NAA treatment (Table 3). Although this effect was not statistically significant in 2010, when averaged across the PGR treatments, the CCC treatment resulted in consistent decreases of AGB compared within the untreated plants (Table 3). This is different from the variable 3-yr seed yield responses, suggesting that the effects of CCC on AGB depend less on climate conditions. The excessive precipitation in both years (2009 and 2011) was probably detrimental to pollination and seed set and activity of bees (Alfalfa leaf – cutting bee), leading to excessive vegetative growth (Iannucci *et al*., 2002; Goldman & Dovrat, 1980; Hutmacher *et al*., 1991).

This result was consistent with the study conducted by Wang (2005), and confirmed that CCC is a useful tool to control alfalfa plant height by reducing active gibberellin levels. However, PGR treatments did not significantly impact plant height (data not shown). The effects of CCC on lodging are more complex, unlike plant height, and resulted in variable responses over 3 year (Fig. 7). Furthermore, lodging could explain the differences in seed yield. As above paragraphs descripted with more precipitation during both years (2009 and 2011), lodging potential was high. Chlormequat chloride application significantly decreased lodging, which compensated for the negative effects of CCC and increased the net seed yield. However, negligible lodging occurred in 2008 and no significant differences were found between CCC treatment and the untreated crop, resulting in a decreased seed yield with the CCC treatment. These results indicated that the different weather conditions primarily accounted for the variable lodging and comparisons of seed yield between CCC treatment and control. Further research is required before the commercial application of CCC can be meaningfully considered in alfalfa seed production.

## Conclusions

Results from the current study provides evidence that: (i) each PGR consistently increased seed yields, and the numbers of seeds per stem compared to untreated plants; (ii) CCC treatment reduced plant height and lodging, but also significantly decreased seed yield. Therefore, we conclude that the effectiveness of CCC application depends on climatic conditions, especially in North-east China. Further study should be conducted to seek optimal management practices to increase yield component and achieve the trade-off of higher quality and greater seed yields under proper management methods. This study failed to identify the optimum combination of CCC with a PGR to increase alfalfa seed production, and none of the PGRs or CCC affected seed quality. But alfalfa seed yield could be improved by combining a PGR such as NAA in North-east China.

## Acknowledgments

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