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Full Length Research Paper

An investigation on mechanisms of blanked nut formation of hazelnut (*Corylus heterophylla* fisch)

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The occurrence of blank nuts is common in *Corylus heterophylla* Fisch orchards of China. This study was aimed to find the possible mechanisms involved in blank nuts formation in wild *C. heterophylla* Fisch species. The effects of pollination, defoliation and girdling on fruit production of *C. heterophylla* Fisch were studied from northern China. The effect of pollination on various aspects of the reproductive output of *C. heterophylla* Fisch was studied by performing hand pollination, open pollination and no pollination. Different pollination types significantly affected flower cluster set including no flower cluster set produced in no pollination treatment. However, pollination type had no direct effect on nut and kernel traits. Three defoliation treatments (control, 50 and 100% leaf removal) were applied at branch level on 10 trees. Six branches were used per treatment in each tree and half of these branches were girdled (a ring of bark and cambium was removed from the branch base). Leaf removal from ungirdled branches had little effect on pistillate flower cluster set, fruit cluster set and nuts per cluster. However, these variables decreased as the extent of 100% defoliation increased on girdled branches. Defoliation and girdling reduced nut and kernel weight which was the result of a reduction in the kernel weight rather than nut coat reduction. Control of the carbohydrate supply to the reproductive shoots by girdling and defoliation made no difference to nut number and size but the kernel percent and blank nut ratio were highly sensitive to carbohydrate availability. Resource importation not exportation by fruiting branches might be a mechanism to reduce blank nut in this species.

Key words: *Corylus heterophylla* Fisch, pollination, defoliation, girdling, blank nut.

INTRODUCTION

Corylus heterophylla Fisch, the Asian Hazel is a species of hazel native to eastern Asia including northern China, eastern Mongolia, Korea, Japan and southeastern Siberia (Whitcher and Wen, 2001). Although the nuts of *C. heterophylla* Fisch have smaller and thicker shells characteristics; the important wild species in China was cultivated commercially for some desirable and economically important traits such as flavor, nonsuckering growth habit, and tolerance to alkaline soil, and exceptionally early maturation and cold hardiness. *C. heterophylla* Fisch is at present an expanding crop in China due to increased demand by the processing industry. Fruit production of hazelnuts mainly depends on the number of female flower setting fruit. Thus, maximizing fruit set is an important measure to increase hazel production. The occurrence of blank nuts is common in *C. heterophylla* Fisch orchards. Shell-kernel weight ratio is the main determinate of quality and price of hazelnuts. The most common defect “blank nuts” in Chinese cultivar have a significant effect on the shell-kernel weight ratio. “Blank” means a filbert containing no kernel or a kernel filling less than one-fifth capacity of the shell. Most species of hazelnut are largely self-incompatible and a number of studies have suggested that self-incompatibility was often associated with a higher frequency of blanks (Erdogan and Mehlenbacher, 2001; Beyhan and Marangoz, 2007). One of the more intriguing aspects of the reproductive biology of hazelnuts is the temporal separation of pollination and fertilization. At the time of pollination, the ovary is not formed and grows only if the flower is pollinated.

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The formation of ovules begins in March and fertilization occurs by the end of May or during the first three weeks of June; two to three months after pollination when the diameter of the nuts is 7 to 10 mm (Germain, 1994). Therefore, different environmental stressors often occur at different stages between pollination and fertilization and lead to poor nut set and higher frequency of blanks (Solar and Stampar, 2001). Hazelnuts fill their fruits in the months of June, July and August. *C. heterophylla* Fisch has high fruit set and large amount of photosynthates are needed during kernel filled stage while photosynthate sinks closest to the leaves tend to be the strongest, so insufficient photosynthates importing into nuts in the filled stage can result in shriveled kernels (Kholupenko et al., 2003). Thus, some authors suggested that the limited resource translocation of carbohydrates from the photosynthetic pool was a possible cause of blank nuts. We investigated possible mechanisms involved in the blank kernels formation in wild *C. heterophylla* Fisch species. The following two hypotheses were set and tested:

H_1: The self-incompatible characteristics in *C. heterophylla* Fisch have an effect on the cluster set of nuts but not the reason of blanked nut.
H_2: The lack of assimilate substances during development of fertilization fruits is the possible cause of blanks and shriveled kernels.

MATERIALS AND METHODS

The study was conducted at an area located in the Siping region (Jilin, China, 43° 09′ 20″ N, 124° 30′ 16″ E) from March 2008 to October 2009. The area has a slope around 5% with a south-western aspect. The adjacent vegetation is mainly natural evergreen forest with some cleared farmland nearby. The *C. heterophylla* Fisch species are grown in the shrub growing form in China, thus the experimental unit was shrub growing system. Samples were collected from five systems (six plants per shrub system). All studied individuals were exposed to sunlight. Plant height ranged from approximately 1.2 to 2.3 m. Pistillate flower clusters (cymule) and fruit clusters of wild *C. heterophylla* Fisch species were examined from 2008 to 2009. The effect of pollination on various aspects of the reproductive output of *C. heterophylla* Fisch was studied by performing hand pollination, open pollination and no pollination. Open-pollinated flowers were collected weekly during the flowering period. For artificial pollination, one to two branches of each tester tree were emasculated by clipping catkins and were covered with Tyvek bags (1*0.5 m) in late March. This was done to isolate female inflorescence and prevent exposure to air-borne pollen. A second Tyvek bag was used to cover and protect the inner bag from damage by wind. Only female flowers from covered branches were used for hand pollination and no pollination test. Abrasion of the styles of flowers by the bag renders them unsuitable for testing. When staminate catkins elongate and are about to shed pollen, they were collected, placed on a sheet of paper in the laboratory and allowed to dry overnight at room temperature (20°C). The following morning the catkins were discarded and pollen was collected and stored in cotton-stoppered vials in the freezer (4°C) for pollination. Hand pollination was made by dusting self-pollen over receptive stigmas with a thin soft brush. No pollination was made on individual branches or the entire trees depending on plant size.

Some unpollinated flowers were collected when styles were visible. Hand pollinated flowers were collected after hand pollination for 24 h. When staminate catkins elongate and are about to shed pollen, they were collected, placed on a sheet of paper in the laboratory and allowed to dry overnight at room temperature (24°C). The following morning, the catkins were discarded and pollen was collected from testers and stored in cotton stopper glass vials at 0°C until used. Some female flowers were pollinated by hand when styles were visible outside the bud or were exerted beyond the red dot stage (>2 mm). The numbers of treated flowers and harvested nut clusters were counted and percent cluster set was calculated as the ratio of nut clusters to flowers pollinated. Pistillate flower clusters were harvested 24 h after hand and no pollination, and styles were processed for fluorescence microscopy for pollen germination and tube growth as follows: for cytochemical assays with bright field and epifluorescence observations using a light microscope, the sampled material was fixed in formalin-aceto-alcohol (FAA) for 48 h and then transferred to 70% ethanol for storage. Fixed samples were then dehydrated in an ethanol series (50, 80, 95, 100, 100%: 12 h each) and transferred to an embedding solvent (xylene; Panreac Quimica SA, Montcada i Reixac, Spain) through a xylene-ethanol series (30, 50, 80, 100, 100%: 12 h each) and finally saturated with paraffin (Paraplast Xtra; Sigma, St Louis, USA). Sections (10 µm thick) were cut with a rotary microtome (Nahita 534; Auxilab SA, Beriain, Spain) and attached to adhesive-treated microscope slides (polysine slides; Menzel GmbH & Co KG, Braunschweig, Germany). Samples were embedded in paraffin, sectioned at 10 mm in a rotary microtome and stained with hematoxylin or safranin-fast green (Odabas, 1976). A girdling treatment was applied to the fruit branch to inhibit the supply of assimilates and/or other substances to the fruit via phloem transport. The effect of this girdling on the occurrence of blanks on the tree was then investigated.

The effect of defoliation and girdling on nut characteristics of *C. heterophylla* was studied after flowering finished. From 28 May 2008, we selected 10 trees for study. After measuring the number of leaves and the fruits on these twigs, 18 tagged shoots per tree were selected for defoliation. Three defoliation treatments (control, 50 and 100% leaf removal) were applied at branch level in 10 trees. Six branches were used per treatment in each tree and half of these branches were girdled. A subset of reproductive shoots was girdled by removing a ring of bark and cambium approximately 1.0 cm wide from the base of the shoot and 5 mm in diameter. This procedure interrupts phloem transport but does not affect xylem transport (Obeso, 1998). Other tagged shoots that were neither defoliated nor girdled, acted as controls. The presence or absence of fruit developed from each flower was recorded so as to determine the fruit set in late-May (the green fruit period just after flowering, hereafter called initial fruit set), in mid-July (the middle stages of seed maturation, hereafter called middle fruit set) and in mid-September (the final stages of seed maturation, hereafter called final fruit set). To examine the effect of assimilate limitation and pollination on fruit traits, the following variables were determined; the green fruits were counted on 28 May as the time for defoliation and girdled. The tagged branches with ripe nuts were harvested on 28 September and the following variables were determined after oven drying; for each sample, the following characters were examined: nuts per cluster, nut and kernel weights, kernel percent (%), shell thickness (mm), good kernel (%) and blank nut (%). In addition, flower cluster drop (%) and fruit cluster drop (%) were examined in the pollination samples.

The design of each experiment was completely randomized with a one-way ANOVA arrangement. Statistical analyses were performed with SAS system 8.0 software and the means were compared using Duncan's multiple range test at 5% level (Duncan, 1955) and values expressed as a percentage were previously

Table 1. Cluster set in different pollinations treatments of hazelnuts for the period of 2008 to 2009.

Year	Treatment	Number of pollination	Pistillate flower cluster drop ratio/percentage	Fruit cluster drop ratio/percentage	Total drop ratio/percentage
2008	Hand pollination	120	10.83±0.41^{e}	29.47±1.15^{b}	40.30±2.10^{e}
	Open pollination	120	31.67±1.50^{c}	22.50±0.96^{d}	54.17±2.65^{c}
	No pollination	60	100.00^{a}	-	100.00^{a}
2009	Hand pollination	120	13.33±0.68^{d}	34.17±1.50^{a}	47.50±2.95^{d}
	Open pollination	120	35.83±1.63^{b}	25.00±1.30^{c}	60.83±3.47^{b}
	No pollination	60	100.00^{a}	-	100.00^{a}
LSD at 0.05			1.95	2.22	3.54

Values are means ± SD. Different letters within a column indicate significant difference at 5% level by Duncan's multiple range tests.

Table 2. Cluster set and frequency of blank nuts at different defoliation and girdling treatments.

Parameter	Control		50% defoliation		100% defoliation		LSD at 0.05
	Ungirdled	Girdled	Ungirdled	Girdled	Ungirdled	Girdled	
Pistillate flower cluster drop ratio/percentage	26.43±1.35^{bc}	25.45±1.58^{c}	28.67±1.35^{ab}	30.32±1.18^{a}	30.09±1.29^{a}	29.43±1.62^{a}	2.64
Fruit cluster drop ratio/percentage	35.26±1.87^{b}	34.23±1.91^{b}	37.73±1.77^{b}	37.56±1.53^{b}	35.39±1.94^{b}	52.27±2.97^{a}	3.85
total drop ratio/percentage	61.69±1.67^{c}	59.68±1.77^{c}	66.4±1.60^{b}	67.88±1.39^{b}	65.48±1.71^{b}	81.7±2.58^{a}	4.29

Values are means ±SD. Different letters within a line indicate significant difference at 5% level by Duncan's multiple range tests.

transformed by calculating the angular transformation.

RESULTS AND DISCUSSION

The different pollination treatment had significant effect on the flower and nut cluster set (Table 1). No flower cluster set was observed in the no-pollinated hazelnut shoots in both years. The total cluster drop varied from 54.17 to 60.83% in both years in the open pollination treatment and there was significant difference for total cluster drop between 2008 and 2009. In *C. heterophylla* Fisch, hand pollination significantly decreased the pistillate flower cluster drop when compared with the open pollination and no pollination treatments but the fruit cluster drop ratio in the hand pollination treatment was higher than the open pollination in both years. Both pistillate flower cluster drop ratio and fruit cluster drop ratio were relatively high in the control treatment, and defoliation and girdling affected both the pistillate flower cluster drop ratio and the fruit cluster drop (Table 2). Defoliation had little effect on the fruit cluster drop of the ungirdled branches but reduced the fruit cluster set on girdled branches especially the final fruit cluster set. Considering the ungirdled branches alone, 50 and 100% defoliation treatment showed no reduced fruit cluster production compared with the control branches. When girdled branches are considered, fruit cluster drop increased from 34.23 to 52.27% as the extent of defoliation increased from 0 to 100 % (Table 2). Beyhan and Marangoz, (2007) reported that cluster droppings were caused by the genetic constitution of the cultivar, alternate bearing habit, pollen source, sexual incompatibility, cultural practices (nutritional deficiencies, lack of irrigation, disease and insect pests) and environmental conditions. According to our result, difference in the percentage of the pistillate flower cluster dropping between different pollination types in the same cultivar was evident. No flower cluster set (initial fruit set) was observed in the no-pollinated hazelnut shoots in both years. Thus, we believed that pollination and fertilization had a direct effect on the flower cluster set.

Thompson (1979) also reported that some ovaries could not grow more than 0.5 mm and these no pollination pistillate flowers dropped in April and May. Thus, lack of fertilization directly led to flower or nut drop. Hand pollination, open pollination and no pollination was performed in the field to verify the effect of pollination and fertilization on the empty of hazelnuts (Table 3 and Figure 1). Fluorescing pollen tubes can be seen at the base of the style after pollinations for 24 h (Figure 1A). We can observe the complete embryo even in the blank nuts (Figure 1F). Hand pollination significantly increased the nuts per cluster compared with the open pollination. But the open pollination was beneficial for the kernel weight and the kernel percent compared to hand pollination

Table 3. Nut and kernel traits in controlled and open pollinations of hazelnuts for the period of 2008 to 2009.

Year	Treatment	Number of nuts per cluster	Nut weight (g)	Kernel weight (g)	Kernel percent (%)	Good kernel (%)	Blank nut (%)
2008	Hand pollination	4.22±0.15^{a}	1.61±0.06^{a}	0.41±0.02^{b}	25.47±0.99^{bc}	60.4±2.35^{a}	44.6±1.54^{a}
	Open pollination	3.57±0.15^{b}	1.45±0.06^{b}	0.36±0.01^{c}	24.83±1.01^{c}	56.7±2.32^{ab}	43.3±1.77^{a}
	No pollination	-	-	-	-	-	-
2009	Hand pollination	4.19±0.15^{a}	1.69±0.06^{a}	0.46±0.02^{a}	27.22±1.27^{ab}	61.2±2.87^{a}	43.8±1.82^{a}
	Open pollination	3.32±0.12^{b}	1.47±0.05^{b}	0.42±0.02^{b}	28.57±1.11^{a}	54.5±2.12^{b}	45.5±1.77^{a}
	No pollination	-	-	-	-	-	-
LSD at 0.05		0.36	0.12	0.03	2.08	5.24	3.27

Values are means ± SD. Different letters within a column indicate significant difference at 5% level by Duncan's multiple range tests.

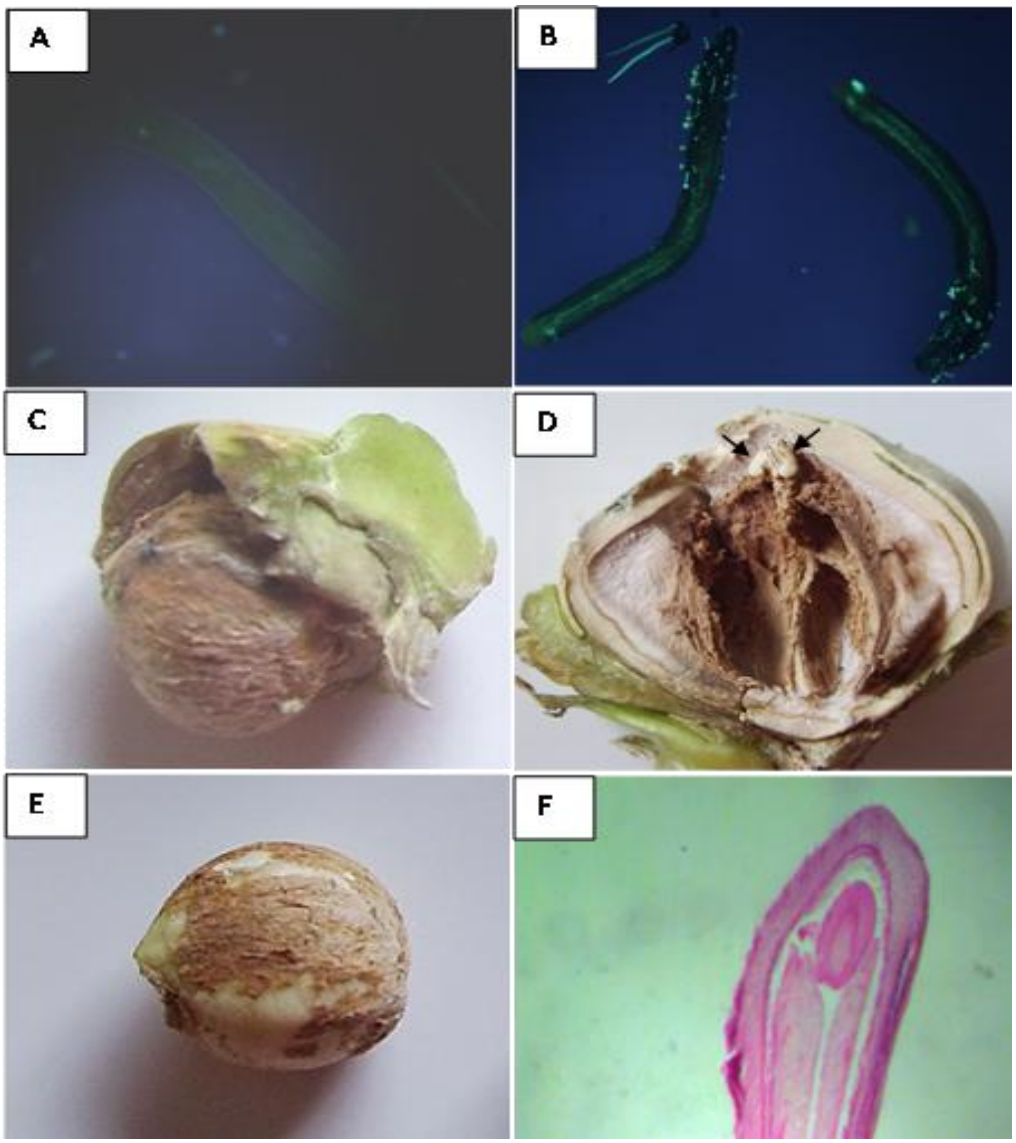

Figure 1. Pollen tubes in a pollinated pistil, and development of full and empty nut of *Corylus heterophylla*. A) control style; B) fluorescing pollen tubes can be seen in style; C) complete embryo could be observed in full nut; D) blank nut, arrow showed the embryo location in blank nut; E) embryo filling the entire ovule; F) complete embryo could be observed in blank nuts (D).

Table 4. Nut and kernel traits at different defoliation and girdling treatments.

Parameter	Control		50% defoliation		100% defoliation		LSD at 0.05
	Ungirdled	Girdled	Ungirdled	Girdled	Ungirdled	Girdled	
Number of nuts per cluster	4.72±0.18^{ab}	4.92±0.24^{a}	4.56±0.15^{bc}	4.62±0.18^{abc}	4.63±0.20^{abc}	4.34±0.20^{c}	0.33
Nut weight (g)	1.45±0.06^{ab}	1.53±0.06^{a}	1.38±0.05^{b}	1.47±0.07^{ab}	1.21±0.06^{c}	1.14±0.05^{c}	0.10
Kernel weight (g)	0.36±0.01^{b}	0.47±0.02^{a}	0.30±0.01^{c}	0.29±0.01^{c}	0.17±0.01^{d}	0.08±0.00^{e}	0.02
Nut coat (g)	1.09±0.04^{b}	1.06±0.04^{b}	1.08±0.04^{b}	1.18±0.06^{a}	1.04±0.05^{b}	1.06±0.05^{b}	0.08
Kernel percent (%)	34.83±1.32^{b}	38.83±1.86^{a}	29.70±0.95^{c}	29.72±1.15^{c}	21.05±0.90^{d}	16.45±0.76^{e}	2.22
Good kernel (%)	46.5±1.95^{b}	59.3±3.44^{a}	41.7±1.54^{c}	44.6±2.18b^{c}	25.3±1.19^{d}	11.3±0.52^{e}	3.39
Blank nut (%)	53.5±2.08^{c}	40.7±1.54^{d}	58.3±2.09^{c}	55.4±2.82^{c}	74.7±3.51^{b}	88.7±4.08^{a}	7.46

Note: values are means ±SD. Different letters within a line indicate significant difference at 5% level by Duncan's multiple range tests.

(Table 3). The ratio of blank nut changed from 43.8 to 45.6% between the hand and open pollination in 2008 and 2009; different pollination model had no significant effect on the blank ratio. A higher frequency of blanks was believed from sexual incompatibility (Erdogan and Mehlenbacher, 2001; Silva et al., 1996) but in our study, no nuts formation can be observed if the flower were not pollinated and fertilized. Furthermore, complete embryo structure can be observed even in the blank nut (Figure 1). So we concluded that unpollinated flower never reaches the size of a blank nut (Thompson, 1967). We could easily see the fluorescing pollen tubes at the base of the style after pollinations for 24 h; hence, we deduced the high frequency of blank nuts not caused by self-pollination.

The results of nut and kernel traits at different defoliation and girdling are shown in Table 4. Girdling and defoliation had little effect on the nuts of per cluster; when 100% defoliated branches were girdled, the branches decreased 9% of their nut production per cluster in proportion to the control ungirdled branches and they produced 88.2% of nut production per cluster in the control girdled branches. The detrimental effect of defoliation and girdling consisted in a reduction of nut and kernel weight which was the result of a reduction in the kernel weight rather than nut coat reduction. The proportion of the good kernel made up by the kernel and nut coat varied among treatments from 16.45 to 38.83%. The ratio of blank nut and kernel percent were significantly affected by defoliation and girdling. In the control treatment, girdling branch produced more kernel percent and low blank nut ratio than girdled branch. But in 50 defoliation treatment, there was no significant different in the kernel percent and blank nut ratio. But in 100 defoliation treatment, the kernel percent (16.45%) and blank nut ratio (88.7%) in the girdled branch was higher than the ungirdled branch (11.3 and 74.7%). Girdling and defoliation and their reaction had little effect on the nuts of per cluster. There was little reduction in nut production in the girdled-100% defoliated branches when compared with the control branches.

There are two alternative explanations for this result: either the nuts of per cluster was not decided by assimilates content but by fertilization or the only sources of assimilates for these branches were the reserves stored in the shoots and/or photosynthesis on green fruits (Hogewoning et al., 2007; Obeso, 1998; Hoch, 2005). Defoliation and girdling and their reaction significantly affected nut mass, which was the result of a reduction in the mass of the kernel rather than the nut coat reduction. Kernel weight proportion of the fruit made up by the kernel mass and nut mass varied among treatment from 16.45 to 38.83%, which means that nut coat was maintained despite whole nut mass reduction (Table 4). When girdling in the control branch was applied, the branches increased the kernel percent and decreased the blank ratio in proportion to the ungirdling branch. However, when 100% defoliated branches were girdled, they produced lower kernel percent (16.45%) and higher blank nut ratio (88.7%) than the ungirdled branch which means that they exported some assimilates to other branches in the cultivation practices (Rivas et al., 2007; Goren et al., 2004). Increase kernel percent and decrease in the blank nut ratio may be the import of resources from other branches rather than export. The ability of resource importation developed by fruiting branches might be a mechanism to increase nut and kernel trait in this species.

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