Gene Manipulation of *Pin* 2(Proteinase Inhibitor II) to the Cottonwood Leaf Beetle(Coleoptera : Chrysomelidae) in Transgenic Poplar(*Populus deltoides* X *P. nigra*)[1]

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形質轉換된 포플러의 딱정벌레에 대한 抵抗性 遺傳子(Proteinase Inhibitor II) 發現[1]

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ABSTRACT

The resistance of a non-transgenic poplar clone, 'Ogy' and three transgenic poplar lines to the cottonwood leaf beetle, *Chrysomela scripta* F., was evaluated by *in vitro* feeding. The lines were transformed with neomycin phosphotransferase II(NPT II) as a selectable marker, proteinase inhibitor II(*pin2*) as a resistance gene, and CaMV 35S as a promoter. An efficient method of sterilizing the beetle eggs and introducing them into plant tissue cultures was developed. The resistance of the transgenic lines was investigated in terms of effects on leaf area consumed, insect weight, insect developmental stages, and plantlet root dry weight after feeding. Also, leaf area consumed was examined by leaf age as measured through leaf plastochron index(LPI). The leaf area consumed and insect weight were highly significant between transformants and control, and insect development *in vitro* was significant among the transgenic lines. Larval infestation was the most severe around LPI 4 to 5 which were young leaves. The system provided a quick, highly controlled method to screen developing transgenic plantlets directly.

Key words : Transgenic poplar, proteinase inhibitor II, cottonwood leaf beetle, in vitro feeding, leaf area consumption, insect weight

要 約

외래 저항성 유전자, Proteinase inhibitor II가 형질전환된 3계통의 벨기에 포플러를 대상으로 딱정벌레에 대한 유전자 발현정도가 기내에서 조사되었다. 포플러 계통은 선발 유전자로서 Nos-promoter 와 Neomycin phosphotransferase gene에 의하여 조절되고 곤충에 대한 저항성 유전자로서 CaMV-35S와 Pin2(Proteinase inhibitor II)에 의한 형질전환체이다. 특히, 형질전환된 포플러의 내충성 저항력을 조기검정하기 위하여, 조직배양을 응용한 새로운 방법으로서 곤충의 알을 표면 살균하여 기내의 조직배양묘와 배양하는 동시배양 방법이 이용되었다. 형질전환된 포플러의 저항성은 기내에서 유충에 의해 섭취된 잎면적, 잎 섭취에 의한 유충의 무게 증감, 유충의 성장단계 등에 의하여 조사되었다. 특히, 잎면적은 각각의 LPI(Leaf plastochron index)별로 측정되었고, 잎면적, 유충의 무게, 곤충의 성장 속도는 형질전환체와 비형질전환체 간에 큰 차이를 보였다. 기내에서 무병상태로 배양된 알들이 부화된 후, 유충의 잎 섭취도는 LPI 4와 5 사이에서 가장 높았다. 본 실험의 기내 배양법은 외래유전자

를 삽입한 이후에 곧바로 유전자 발현을 빠른 시간내에 조기검정 할 수 있는 새로운 방법의 개발이라 할 수 있다.

INTRODUCTION

The genus *Populus* has many advantages as a model system for biotechnological research in trees because it contains a wide genetic diversity, many species hybridize readily, and most sections propagate relatively well(Kang and Hall, 1996). *Populus* species are grouped into three primary sections : Leuce(aspen), Aigeiros(cottonwood), and Tacamahaca(balsam poplar). The poplars are a focus for short-rotation biomass-energy research(Hall et al., 1991 ; Ranney et al., 1985).

The cottonwood leaf beetle, *Chrysomela scripta* F.(Coleoptera : Chrysomelidae), is a multivoltine insect considered to be the major leaf defoliator of *Populus* plantations(Burkot and Benjamin, 1979 ; Harrell, 1980). Defoliation reduces height growth as well as promoting access by secondary pathogens(Kulman, 1971 ; Rickenbacker, 1994). The beetle prefers succulent tissue containing high concentrations of nutrients(Bingaman and Hart, 1992 ; Harrell et al., 1982).

In natural populations, the cottonwood leaf beetle exhibits non-preference for Leuce(aspen spp.) whereas the insect shows preference for Aigeiros and Tacamahaca sections(Caldbeck et al., 1978). Most *Populus deltoides* clones and many hybrids from the Aigeiros section were preferred in multiple choice tests(Bingaman and Hart, 1992). Several papers have been reported the preference of the beetles for different clones (Bassman et al., 1982 ; Bingaman and Hart, 1992 ; Caldbeck et al., 1978 ; Harrell et al., 1981). To test clonal preference, Harrell et al. (1981) selected 12 hybrid poplar clones. They suggested that the adult beetle exhibited non-preference for the Leuce section. They preferred 100% Tacamahaca to 100% Aigeiros. However, the hybrids derived from these sections showed various levels of infestation. Tissue-cultured plantlets have been used in *ex vitro* tests of beetle preference with *Populus euramericana* hy-

brid clones(Harrell et al., 1982). *In vitro* insect colonization studies have been reported with spruce budworm(Retnakaran and French, 1971) and northern fowl mites(Carroll et al., 1992).

To improve resistance of trees by biotechnological methods, the focus has been on antibiosis, in which the biology of the pest insect is adversely affected. So far, two strategies have been considered in *Populus* species : (1)endo-toxin genes from *Bacillus thuringiensis*(B.t.), (2)proteinase inhibitor genes(PI) from potato. *Bacillus thuringiensis* is a gram-positive soil bacterium characterized by its ability to produce crystalline inclusions during sporulation in the midgut of insects. The insecticidal toxins are relatively pest-specific with different strains of *B.t.*, affecting lepidopterans(Knowles and Ellar, 1988), coleopterans(Herrnstadt et al., 1986), and dipterans(Tyrell et al., 1979). The *B.t.* endotoxin gene has been transformed into the 'Crandon' clone(*Populus alba* X *P. grandidentata*) and *P. nigra* x *P. trichocarpa* by a method of electric discharge particle acceleration(McCown et al., 1991).

Proteinase inhibitors are small proteins which occur naturally in plants, animals, and microorganisms, that inhibit the action of digestive enzymes in the insect midgut(Ryan, 1989). Proteinase inhibitors in plants are used as a defense from herbivores and adversely affect insect growth(Broadway et al., 1986). The transformation systems with proteinase inhibitor gene(*pin2*) were conducted in the 'Hansen' and 'Crandon' clones(*Populus alba* X *P. grandidentata*) and the 'Ogy' clone(*Populus deltoides* X *P. nigra*) by an *Agrobacterium* binary vector system(Chun et al., 1988 ; Heuchelin et al., 1991 ; Klopfenstein et al., 1993). The 'Ogy' clone is a hybrid introduced from Belgium, where it was selected for its fast growth and good wood quality(Steenackers, 1987).

An *in vitro* feeding method using *in situ* plants has not been reported for the early selection of transgenic plants. The objectives of this

study were to evaluate the potential of the proteinase inhibitor gene II(*pin2*) to confer subtle resistance to a major pest of *Populus* species and the feasibility of using tissue-culture materials *in situ* for more rapid screening of transformed materials. This experiment was conducted as a no-choice test in Magenta plastic cages which have the advantage of protecting test insects from predation and parasitism.

MATERIALS AND METHODS

Plant Materials

The base clone for the study was 'Ogy'(*Populus deltoides* X *P. nigra*) which is a preferred host for the cottonwood leaf beetle. The heaviest damage occurs during the larval stage in the insect life cycle. The 'Ogy' clone was selected at the Poplar Research Center in Geraardsbergen, Belgium(Steenackers, 1987).

Three transformed(Tr 3A, 5B, and 10A) lines of 'Ogy' were available from a previous study (Heuchelin et al., 1991). The transgenic lines were produced from leaf culture with a binary vector of *Agrobacterium tumefaciens* containing the plasmid pRT104 and a disarmed EHA101 helper plasmid. The plasmid pRT104(17.0kb) was composed of the NPT II selectable marker with the NOS promoter, CaMV-35S promoter, and the proteinase inhibitor II(*pin2*) in the T-DNA region(Fig. 1). Proteinase inhibitors are small proteins that block the functional gene with a CaMV-35S promoter producing trypsin-like-enzymes in the digestive system in insects. In previous studies, the selected lines were tested for the expression of the proteinase inhibitor gene II(*pin2*) by Southern and Western blots. Among the transgenic lines, Tr 5B was the most consistent transformant(Heuchelin, 1992).

The stock plants of the 'Ogy' clone and the three transgenic lines were maintained at 25℃ under natural day light and fluorescent night light(16 : 8) in the Iowa State University Forestry greenhouse. The stock plants were treated weekly with fertilizer(N : P : K=20 : 10 : 20).

In vitro Establishment for Insect Feeding

Stem segments(2-month-old) containing axillary buds from 'Ogy' parental and transformed lines were collected from actively growing greenhouse stock plants. The outer leaves were removed from the collected buds and the buds were rinsed with tap water. The explants were dipped in 70% ethanol for 1 minute, sterilized in a solution of 2% sodium hypochlorite for 40 minutes and finally rinsed three times with sterilized deionized water. The explants were cultured in test tubes(2.4×15cm) on MS(Murashige and Skoog, 1962) medium containing N and N vitamin mix. (Nitsch and Nitsch, 1969) without plant growth regulators. After developing shoots were proliferated, three elongated shoots were transferred to each Magenta GA-7 vessel(7.6×7.6×10.2cm, Magenta Corp. Chicago IL) with 80ml of the medium containing 0.02mg/l NAA(Naphthaleneacetic acid) for shoot elongation. To maximize the room for plant expansion *in vitro*, two Magenta vessels were combined by means of a coupler. After 2 months, the fully elongated shoots served as the source of feeding materials under *in situ* conditions. Leaves on the cultured plants were identified using the Leaf Plastocron Index(LPI) system(Larson and Isebrands, 1971). The top developing leaf(less than 20mm) was designated LPI 0. The numbers of each LPI were then assigned from the apex to base.

Insect Rearing and In vitro Sterilization

Adults of the cottonwood leaf beetle were collected from a central Iowa plantation in late May

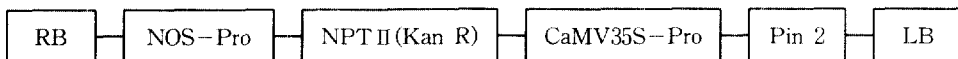| RB | NOS—Pro | NPT II (Kan R) | CaMV35S—Pro | Pin 2 | LB |

Fig. 1. Diagramatic T-DNA map of the transforming plasmid, pRT 104. The plasmid contains NPT II(neomycin phosphotransferase II) consisting of the Nos(nopaline synthase) promoter, pin2 (potato proteinase inhibitor II) consisting of the CaMV 35S promoter. The total size of plasmid is 9.0kb.

and in early July, 1994. The adults(20 to 30 individuals) were reared in crispers(26.4×19.2× 9.5cm) in a culture room at 23±2℃. Succulent leaves of less than LPI 10 from *Populus deltoi-des*(pure lines) were used as food sources for the colony. Fresh leaves were provided to the beetles with a period of every 2 or 3 days. The colony was monitored for signs of contamination. For each experiment, a new colony of beetles was established from field collections. Egg masses were used to initiate co-cultures with the *in vitro* plant materials. The egg masses were separated by treatment with sodium hydroxide(1.0%) and shaking for 10 minutes. The separated eggs were rinsed with distilled water. The eggs were disinfected by soaking in sodium hypochlorite (0.1%) for 1 min. Finally, eggs were rinsed four times with distilled water and surface dried on sterilized filter paper under a laminar air-flow hood. After they were completely dry, the separated eggs(8 to 10) were inoculated onto the LPI 4 leaf of test plantlets using a sterilized paint brush. The *in vitro* cultures were continued for 12 days after egg placement at 23±2℃ with a photoperiod of 16L : 8D. After 12 days, both test plantlets and feeding insects were collected for measurement of leaf area consumption and insect weight. Total amounts of leaf area consumed from each LPI leaf were measured by a Delta T area meter(model MK-2). Simultaneously, insect developmental stages were recorded and root dry weights also were measured after the feeding had occurred.

The experiment was conducted twice with a total of 5 replications of the transgenic lines and 8 replications of the parental line. A replication consisted of 3 plantlets of a single line in a Magenta box. Data were analyzed for leaf area consumption, insect weight, insect developmental stages, and root dry weight by the general linear model(GLM) of analysis of variance.

RESULTS AND DISCUSSION

After two months, plantlets were fully elongated on MS medium in coupled Magenta GA-7 vessels. Sodium hydroxide(1.0%) and sodium hypochlorite(0.1%) worked well for separation and disinfection of eggs of the cottonwood leaf beetle. The hatching rate from sterilized eggs was about 63%. Some eggs did not hatch due to damage during the inoculating procedures on the leaves of test plantlets. In preliminary tests, ethanol(70%) was applied to promote disinfection of eggs for 1 minute. However, most eggs were killed by this treatment. A sterilization method for cottonwood leaf beetle has been previously reported(Bauer, 1990). However, the concentrations of both chemicals used were so low that the contamination from microorganisms was not entirely eliminated.

The parental and transgenic lines were tested in preliminary bioassays with the cottonwood leaf beetle. Factors affecting *in vitro* feeding experiments might be insect density, plantlet maturation, feeding time, and environmental conditions such as photoperiod and temperature. From the preliminary tests, the density of larva and the duration of *in vitro* feeding were determined for the main experiments. A density of 8 to 10 larva was suitable with a period of 12 days feeding. An occasional larva would fall off a leaf, stick to the agar, and die. Therefore, results were based on the number of larvae surviving at the end of the experiment. The results of mean leaf area consumption were significantly different($P < 0.0001$) among the plant lines and by LPI($P < 0.0001$) of each plant source(Fig. 2). After sterilized eggs hatched *in vitro*, the larvae started to feed on the leaves around LPI 4 and then moved onward to other succulent leaves. The patterns of infestation appeared heavier around LPI 4 where the eggs were placed(Fig. 2). The parental(non-transgenic) line seemed to have a wider range of infestation across the LPI. Similar results were reported from *ex vitro* feeding studies of cottonwood leaf beetle(Bingaman and Hart, 1992 ; Harrell et al., 1982). Fig. 3 shows the mean total leaf area consumed from each of the plant lines('Ogy', 1275.50±43.70 ; Tr 3A, 759.10±62.47 ; 5B, 730.67±60.29 ; 10A, 696.94±49.0mm^2 per insect). The least infestation occurred in Tr 10A, at half the level of the parental line 'Ogy'. There were only small dif-
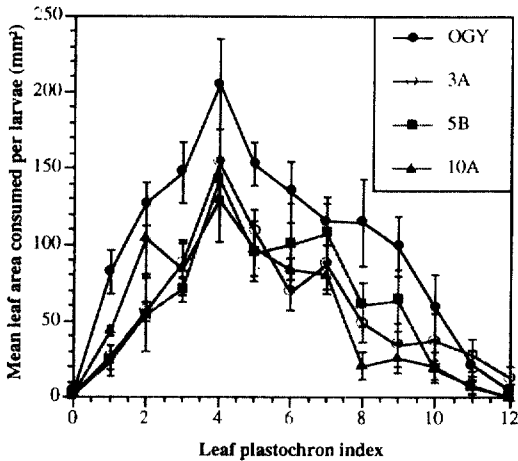
Fig. 2. Mean leaf area consumed per larvae of cottonwood leaf beetle on the 'Ogy' clone as tested by LPI. The vertical bars on the graph represent the standard error.
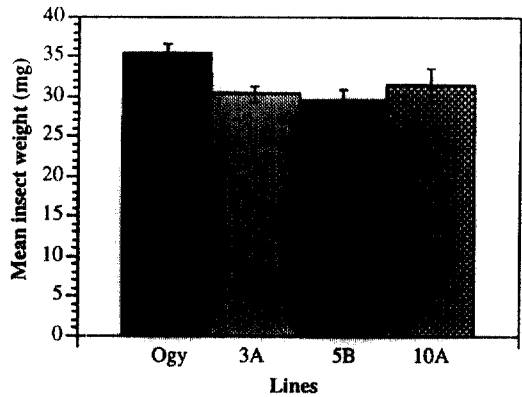

Fig. 3. Mean total leaf area consumed per larvae after 12 days feeding on two-month-old 'Ogy' clone plantlets. The vertical bars on the graph represent the standard error.

ferences among transgenic lines in leaf area consumed.

After larvae fed for 12 days, the weights of insects were measured(N=105 insects). The mean weights were significantly different(P<0.0022) among the feeding sources. Also, there were significant differences(P<0.03) among the replications. Fig. 4 represents the mean weights of insects after the feeding on each line('Ogy', 35.41±1.09 ; Tr 3A, 30.26±1.08 ; 5B, 29.41± 1.42 ; and 10A, 31.44±2.13mg). In terms of

Fig. 4. Mean insect weight of cottonwood leaf beetle after 12 days feeding on the 'Ogy' clone and its transgenic derivatives. The vertical bars on the graph represent the standard error.
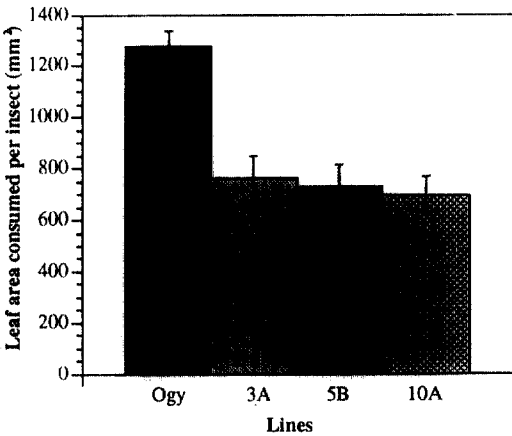
insect weight, Tr 5B seemed to have the most effect. The larva consumed more food from the plantlets of the parental line(non-transgenic), and probably were digesting it more thoroughly than was the case with the transgenic lines.

Insect developmental stages were analyzed in the experiment. In preliminary experiments, insect stages were observed from *in vitro* feeding tests with the 'Ogy' clone. Surface sterilized eggs hatched *in vitro* three days after initial inoculation. Larva and prepupa stages took nine and three days, respectively. Therefore, 12 days was chosen as the duration of feeding for the main experiment. These developmental stages were used to analyze insect maturation. The developmental stages were also significantly different(P<0.038) among the insects of each transgenic line tested. It was highly significant (P<0.0001) within the replications of the lines. Each stage of the insect tested was coincided simultaneously within the lines when the feeding was finished. When data of insect developmental stage were collected, each stage was recorded as 3 for larvae, 12 for prepupae, and 15 for pupae as developmental days. Fig. 5 represents the mean insect developmental stages in each transgenic lines('Ogy', 10.84±0.94 ; Tr 3A, 9.25± 0.96 ; 5B, 9.05±1.0 ; 10A, 8.0±1.3 days). By the mean data of insect developmental stages, every line containing non-transgenic line was
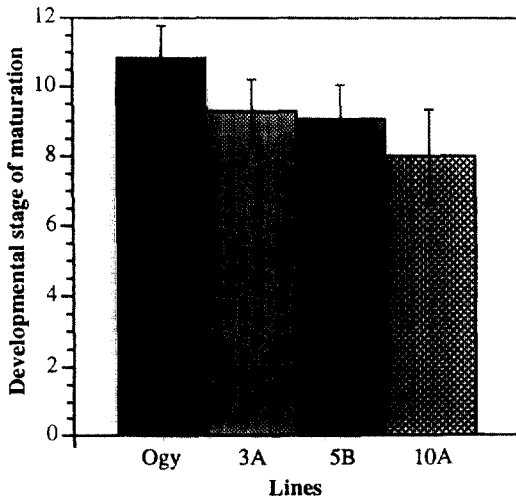
Fig. 5. Mean insect developmental stage after 12 days feeding with two-month-old tissue cultured plantlets. The vertical bars on the graph represent the standard error.

under prepupae stage. Among the transgenic lines, the insect development was much more suppressed in Tr 10A theoretically due to blocking of the enzymatic system of the insect midgut.

After the feeding data were collected from the *in vitro* experiment, root dry weights were measured. Differences were only significant at the level($P < 0.088$) among the transgenic lines. The weights were heavier in transgenic lines than in the parental line with or without feeding. Transgenic line Tr 10A had the heaviest root weight (0.30 ± 0.09g) compared to 'Ogy' with feeding (0.17 ± 0.08g) or 'Ogy' without feeding(0.18g). These results probably reflect the lower infestation rate on the transgenic lines. However, there were insufficient transgenic plantlets to run a direct comparison between the effects of feeding and non-feeding on root weight.

For the preference of feeding and oviposition, mechanisms are classified into physical and chemical characteristics of host plants. Leaf morphology(age, shape, color, and trichomes) and nutritional values(water, sugar contents) all affected the selection of hosts by herbivorous insects(John, 1975 ; Raupp, 1985). Conventionally, clonal preferences of the cottonwood leaf beetle

have been studied in the *ex vitro* condition (Caldbeck et al., 1978 ; Meyer and Montgomery, 1987 ; Wilson, 1979) and on laboratory bioassays (Harrell et al., 1981). These previous results showed that the Aigeiros section of *Populus* was highly susceptible whereas the Leuce section (aspen) and the aspen hybrids were not preferred for feeding and oviposition. Young succulent leaves were more preferred for host selection because they contained more nutrition and less defensive chemicals. Secondary metabolites such as phenolics and phenolic glycosides in *Populus* are used as defense chemicals against insect pests(Harborne, 1985 ; Levin, 1971). The amount of phenolic glycosides in *Populus* clones have been quantitatively assayed in quaking aspen (*Populus tremuloides*)(Lindroth and Hemming, 1990), *Populus deltoides*, and some hybrids(Bingaman and Hart, 1993). These papers suggested that the high concentrations of glycosides preferred for feeding and oviposition.

Succulent leaves regenerated from tissue culture previously have been tested in *ex vitro* trials for feeding preference(Burkot and Benjamin, 1979 ; Harrell et al., 1982). The results suggested that adult beetles preferred the younger, more succulent leaves. Similar feeding techniques were conducted with *ex vitro* conditions with transgenic lines of 'Crandon' and 'Hansen' clones(*Populus alba* x *P. grandidentata*) to evaluate their resistance to different insects(Allen, 1992 ; McCown et al., 1991). Transgenic lines of 'Crandon' expressing CaMV 35S-BT(*Bacillus thuringiensis*) were tested for lepidopteran insects (forest tent caterpillar and gypsy moth). The larva survival and the larva weight gain were significantly decreased by the feeding of transgenic plants(McCown et al., 1991). Also, transgenic 'Hansen' and 'Crandon'(*Populus alba* x *P. grandidentata*) producing proteinase inhibitor II (*pin2*) were tested with the willow leaf beetle by single and multiple generation tests(Allen, 1992). The levels of infestation, pupae weight, and insect developmental time were shown to differ among the tested host lines.

For the selection of transgenic plants, we developed a new technique. For the first time, we

report an *in vitro* feeding method with *in situ* conditions for more rapid screening with transgenic plantlets. The results suggest that this method is feasible for screening of transgenic poplars over shorter time periods with more environmental control. Of course, to be fully validated, the technique needs to be compared to field tests of the same transgenic lines.

LITERATURE CITED

1. Allen, K.K. 1992. Effects of transgenic *Populus* on the biology of *Plagiodera versicolora*(Laicharting, Coleoptera : Chrysomelidae). M.S. Thesis. Iowa State University, Ames, Iowa, 50011.

2. Bassman, J., W. Myers, D. Dickmann, and L. Wilson. 1982. Effects of simulated insect damage on early growth of nursery-grown hybrid poplars in northern Wisconsin. Can. J. For. Res. 12 : 1-9.

3. Bauer, L.S., J. Meerschaet, and O.T. Forrester. 1990. An artificial diet for cottonwood and imported willow leaf beetles (Coleoptera : Chrysomelidae) and comparative performance on poplar foliage. J. Entomol. Sci. 25(3) : 475-480.

4. Bingaman, B.R. and E.R. Hart. 1992. Feeding and oviposition preferences of adult cottonwood leaf beetles(Coleoptera : Chrysomelidae) among *Populus* clones and leaf age classes. Environ. Entomol. 21 : 508-517.

5. Bingaman, B.R. and E.R. Hart. 1993. Clonal and leaf age variation in *Populus* phenolic glycosides : Implications for host selection by *Chrysomela scripta*(Coleoptera : Chrysomelidae). Environ. Entomol. 22(2) : 397-403.

6. Broadway, R.M., S.S. Duffey, G. Pearce, and C.A. Ryan. 1986. Plant proteinase inhibitors : A defense against herbivorous insects? Entomol. Exp. Appl. 41 : 33-38.

7. Burkot, T.R. and D.M. Benjamin. 1979. The biology and ecology of the cottonwood leaf beetle, *Chrysomela scripta*(Coleoptera : Chrysomelidae), on tissue cultured hybrid Aigeiros(*Populus* X *euramericana*) subclones in Wisconsin. Can. Entomol. 111 : 551-556.

8. Caldbeck, E.S., H.S. McNabb, Jr., and E.R. Hart. 1978. Poplar clonal preferences of the cottonwood leaf beetle. J. Econ. Entomol. 71 : 518-520.

9. Chun, Y.W., N.B. Klopfenstein, H.S. McNabb, Jr. and R.B. Hall. 1988. Transformation of *Populus* species by an *Agrobacterium* binary vector system. J. Kor. For. Soc. 77 : 199-207.

10. Hall, R.B., E.R. Hart, H.S. McNabb, Jr., B.G. McMahon, and R.D. Hanna. 1991. Selection and breeding of pest-resistant clones of *Populus* for biomass energy production in the north central region. SAE Technical Paper. Detroit, MI.

11. Harborne, J.B. 1985. Phenolics and plant defense, pp.393-408. *In* : C.F. Van Sumere and P.J. Lea [eds.], The phytochemical society of Europe. Carendon, Oxford.

12. Harrell, M.O. 1980. The nutritional ecology of the cottonwood leaf beetle as influenced by hybrid poplar clonal foliage, with notes on the insects associated with tissue-cultured hybrid poplars in Wisconsin. Ph.D. .dissertation, University of Wisconsin, Madison.

13. Harrell, M.O., D.M. Benjamin, J.G. Berbee, and T.R. Burkot. 1981. Evaluation of adult cottonwood leaf beetle, *Chrysomela scripta*(Coleoptera : Chrysomelidae), feeding preference for hybrid poplars. The Great Lakes Entomol. 14(4) : 181-185.

14. Harrell, M.O., D.M. Benjamin, J.G. Berbee, and T.R. Burkot. 1982. Consumption and utilization of leaf tissue of tissue-cultured *Populus* X *euramericana* by the cottonwood leaf beetle, *Chrysomela scripta*(Coleoptera : Chrysomelidae). Can Ent. 114 : 743-749.

15. Hermstadt, C., G.G. Soares, E.R. Wilcox, and D.L. Edwards. 1986. A new strain of *Bacillus thuringiensis* with activity against coleopteran insects. Bio/Technology, 4 : 305-308.

16. Heuchelin, S.A., H.S. McNabb, Jr., N.B. Klopfenstein, and R.W. Thornburg. 1991. *Agrobacterium* mediated transformation of *Populus* X *euramericana* 'Ogy' using the proteinase inhibitor II gene(*pin2*) to increase

pest resistance. Phytopathology 976.

17. Heuchelin, S.A. 1992. Transformation of *Populus* X *euramericana* 'Ogy' with the *pin2* gene. M.S. Thesis. Iowa State University, Ames, IA 50011.

18. Johnson, H.R. 1975. Plant pubescence : An ecological perspective. Bot. Rev. 41 : 233-258.

19. Kang, H. and R.B. Hall. 1996. Light and scanning electron microscope observation of shoot formation in developing ovules of cottonwood(*Populus deltoides*). Kor. Plant Tissue Culture 23(2) : 83-88.

20. Klopfenstein, N.B., H.S. McNabb Jr., E.R. Hart, R.B. Hall, R.D. Hanna, S.A. Heuchelin, K.K. Allen, Nian-Qing Shi, and R.W. Thornburg. 1993. Transformation of *Populus* hybrids to study and improve pest resistance. Silvae Genetica 42 : 86-90.

21. Knowles, B.H. and D.J. Ellar. 1988. Differential specificity of two insecticidal toxins from *Bacillus thuringiensis* var. *aizawai*. Mol. Microbiol., 2(1) : 153-157.

22. Kulman, H.M. 1971. Effects of insect defoliation on growth and mortality of trees. Annu. Rev. Entomol. 16 : 289-324.

23. Levin, D.A. 1971. Plant phenolics : An ecological perspective. Am. Nat. 105 : 57-181.

24. McCown, B.H., D.E. McCabe, D.R. Russell, D.J. Robison, K.A. Barton, and K.F. Raffa. 1991. Stable transformation of *Populus* and incorporation of pest resistance by electric discharge particle acceleration. Plant Cell Reports 9 : 590-594.

25. Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15 : 473-497.

26. Nitsch, J.P. and C. Nitsch. 1969. Haploid plants from pollen grains. Science 163 : 85-87.

27. Ranney, J.W., L.L. Wright, J.L. Trimble, R.D. Perlack, D.H. Dawson, C.R. Wenzel, and D.T. Curtin. 1985. Short Rotation Woody Crops Program : Annual Progress Report for 1984. ORNL-6160. Oak Ridge National Laboratory, Oak Ridge, TN.

28. Raupp, M.J. 1985. Effects of leaf toughness on mandibular wear of the leaf beetle, *Plagiodera versicolora*. Ecol. Entomol. 10 : 73-79.

29. Reinchenbacker, R.R. 1994. The impact of artificial defoliation of *Populus* growth, biomass production, and total non-structural carbohydrate concentration. M.S. Thesis. Iowa State University, Ames, Iowa 50011. Ryan, C.A. 1989. Proteinase inhibitor gene families : Strategies for transformation to improve plant defenses against herbivores. BioEssays 10 : 20-24.

31. Steenackers, V. 1987. Government Poplar Research Station Activity Report-Recent Advances 1987. In Activity Report of the Belgium National Poplar Commission to the 18th session of the International Poplar Commission. Beijing-China, 5-8 September 1988. Published by Belgian National Poplar Commission, Bolwerklaan 21, 1210 Brussel Belgium. pp.63-75.

32. Tyrell, D.J., L.I. Davidson, L.A. Bulla, Jr., and W.A. Ramoska. 1979. Toxicity of parasporal crystals of *Bacillus thuringiensis* subsp. israelensis to mosquitoes. Appl. Environ. Microbiol., 38 : 656-658.

33. Wilson, L.F. 1979. Insect pests of *Populus* in the lake states, pp.75-81. *In* : Proceedings, 16th Annual Mtg. North American Poplar Council, Thompsonville, MI.