IJSRM
INTERNATIONAL JOURNAL OF SCIENCE AND RESEARCH METHODOLOGY
An Official Publication of Human Journals

Human Journals
Research Article
March 2018 Vol.:9, Issue:1

Natural Cytokinin and Abscissic Acid (ABA) Hormones Isolation from Peach Plant and Its Effect as Growth Regulator on Callus Cell *in Vitro* and Phloem Tissue *in Vivo*

IJSRM
INTERNATIONAL JOURNAL OF SCIENCE AND RESEARCH METHODOLOGY
An Official Publication of Human Journals

A. B. M. Sharif Hossain[1,2*]

1 Biotechnology Program, Department of Biology, Faculty of Science, University of Hail, KSA

2 The University Experimental Farm, Department of Bioresource Production Science, The United Graduate School of Agricultural Science, Ehime University, Hattanji 498, Matsuyama shi 799-2424, Japan

Submission: 26 February 2018
Accepted: 4 March 2018
Published: 31 March 2018

Keywords: Abscissic Acid, Cytokinin, Peach Leaf and Tissue, Callus Cell

ABSTRACT

Phytohormone is a natural plant derived chemical that regulates cell, tissue and plant growth. They are signal molecules produced within the cell, tissue as a whole in the plant. Plant hormones e.g. cytokinin and abscissic acid (ABA) regulate cellular processes in certain cells and moved to different locations of the plant parts. They enhance the formation of cell, tissue, leaves, stems, flowers and fruit. The study was carried out to isolate cytokinin and ABA from young leaves and roots of peach trees as affected by Partial Phloem Cut Ring (PPCR) and Complete Phloem Cut Ring (CPCR). From the results, it had been found that phloem tissue growth was higher in the PPCR and control than in CPCR treated trees. Free ABA, bound ABA and total ABA content of peach shoot leaves were higher in CPCR and PPCR than control trees (no phloem cut). Phloem tissue growth was found 11.1 and 13.2% in PPCR and CPCR followed by 6.1% in the control trees (no phloem cut). Moreover, cytokinin was found better effect on callus cell growth in control than phloem cut. The results showed that PPCR and CPCR increased ABA content in leaves and control increased the cytokinin content.

INTRODUCTION

Cytokinin is a plant growth hormone or phytohormone that promotes cell division or cytokinesis in cell and tissues of plant roots and shoots (Kaiber 2002). Mullins (1967) stated that cytokinin successfully stimulated for root growth of young grapes. Antognozzi *et al*. (1993) reported that cytokinin activating compound N_1-(2-chloro-4pyridyl)-N_3-phenylurea, CPPU increased the transverse diameter, size and fresh weight of olives. Park *et al*. (1997) observed that stem growth of kinetin treated persimmon trees was higher than control trees. Arakawa *et al.* (1997) reported that stem growth of apple trees above phloem cut was significantly increased and below phloem cut stem growth was reduced. Jose (1997) found lower vegetative growth in girdling treatments in relation to control in mango trees. Onguso *et al.* (2004) reported that the increase of trunk circumference above girdling might be caused by swelling of trunk with accumulation of carbohydrates. They also stated that girdling blocked the translocation of sucrose from leaves to roots through phloem bundles. The block decreased starch content in root system and accumulated of sucrose in the leaves.

ABA is a plant hormone and a single compound, unlike the auxins, gibberellins, and cytokinins. It was thought to play a major role in abscission of fruits (Mauseth, 1991). ABA plays an inhibitory role, it has many promoting functions as well (Raven, 1992; Salisbury and Ross, 1992). Lignin content increased in one-year-old peach shoot sprayed with ABA and made the cell, tissue and trees dwarfed (Khamis and Holubowicz, 1978). Increase of Endogenous ABA has been demonstrated for several stress phenomena like salinity, relative humidity, osmotic root stress and wilting (Wright and Hiron, 1969). It was observed that accumulation of ABA indeed reflected the tissue response to senescence-inducing stimuli (Goldschmidt *et al*. 1973). Hossain and Uddin (2017) reported that it was possible to make peach phloem tissue greatly inhibited by using ABA, 1000 and 2000ppm applied to the bark phloem tissue when compared to the water control (Hossain *et al*. 2007; Hossain and Mizutani, 2009). It has been reported that anticancer properties was supported by using ABA (Gonzalo, 2014). Zhao *et al* (2007) observed that plant stress hormone ABA suppressed the proliferation and induced apoptosis in human cancer cell (Livingston, 1984).

There are not available literature on natural ABA and cytokinin isolation and its effects in this regards. That is why this study was undertaken to identify the ABA component as inhibitor and to isolate and obtain a quantitative estimation of the changes which occurred

during senescence as affected by phloem cut on the stem. In addition, to isolate the cytokinin content and used as promoter using soybean callus formation.

MATERIALS AND METHODS

Experiment 1

Plant materials

The experiment was carried out in an orchard in the Ehime University Farm located in southern Japan. One-years-old peach (*Prunus persica* Batsch cv. 'Hikawahakuho') trees grafted on peach seedling stocks (wild form) were used in this experiment. The seedling rootstocks were collected from nursery and transplanted to the main field. The plant was spaced at 0.60 m x 1.0 m. Weeding was done by maintaining row as required during the plant vegetative stage for 5 months. Granular fertilizers were applied after transplanting at the rate of N, P_2O_5 and K_2O 10 g, 10 g and 10 g per tree respectively. Irrigation was applied once in a week by hosepipe. Insecticide was applied once in a month.

Treatment set up

Treatments were set after15 days of seedling growth. Partial phloem cut (Partial Ring, PR) was done by using a small sharp knife removing a partial phloem 2 cm length leaving a connecting strip (bridge) 2 mm width (thickness) in the trunk 20 cm above from the ground. In case of complete phloem cut (Complete Ringing, CR) there was no connecting bridge or strip. There were 3 treatments each with 4 replications used in different trees in the experiment. For each replication, each individual tree was used. There were a total 12 (4 x 3) trees used for the experiment. The treatments were control (no phloem cut) partial ringing (Partial phloem cut) and complete ringing (complete phloem cut).

Sample collection and preparation

Young leaf samples were collected after two months (July 10) of treatment application All trees were uprooted and washed. Fresh leaves were separated, washed and kept in the Freezer immediately after harvest and used for ABA analysis.

ABA isolation

The analysis was carried out according to the method of Most *et al*. The samples were homogenized in 80% ethanol and filtered. They were then concentrated *in vacuo* to aqueous phase using a rotary evaporator. The aqueous phase was mixed with insoluble PolyVinyl Pyrollidone (PVP) (500 mg/5ml H_2O) and filtered. The pH of the filtrate was adjusted to 8.5 using 5% NH_4OH followed by partitioning with 10 ml of CH_2Cl_2. The partitioning was repeated four times following the same procedure**.**

Organic phase

The organic phase was discarded and the pH of the aqueous phase was adjusted to 3 using 1 N HCl. Partitioning was again repeated four times with CH_2Cl_2 as explained above. The organic phase was partitioned four times with 10 ml of bicarbonate buffer (CO_3^{2-} buffer, pH 10) and the alkaline aqueous phase was collected. The organic phase including free ABA evaporated to dryness in vacuo.

Aqueous phase

For hydrolysis of bound ABA, the pH of the acidic aqueous was adjusted to 10.5 with 5% NH_4OH and the samples were heated in a water bath at 60ºC for 45 min. The solution was left to cool at room temperature for 1 h and the pH was adjusted to 3 with 1 N HCl and partitioned four times with CH_2Cl_2. The organic phase was retained and evaporated to dryness in vacuum.

Methylation of ABA and GC conditions: The samples were dissolved in 5 ml acetone/methanol (9/1) and the methylation with diazomethane carried out as shown in Fig. 1, was modified from Schlenk and Gellerman [13]. The test tubes were arranged as shown with tube 1 containing acetone, tube 2 carbitol, KOH and NMSA (n-methyl-n-nitroso-p-toluenesulfonamide) (5 g/50 ml acetone) and tube 3 the sample dissolved in acetone/MeOH. Nitrogen gas was passed through the sample for 3 min. The sample was evaporated overnight at room temperature with the help of a fan. The sample was taken up in 1ml acetone and 1 µl injected into a Gas Chromatograph (GC-8A, Shimadzu, Kyoto, Japan) equipped with an electron capture detector (^{63}Ni) and a glass column packed with Gaschrom Q (80-100 mesh) coated with 2% silicon OV-7. The injection/detector and column temperatures were 240 ºC and 230 ºC, respectively. The flow rate of the carrier gas (N_2) was 40 ml/min.

Experiment 2

Plant materials

Same as mentioned in Experiment 1.

Treatment set up

Same as mentioned in Experiment 1.

Sample collection and preparation

All trees were uprooted and washed. Root samples (30 g) were collected after two months of treatment setting. Fresh roots were separated, washed and kept in the Freezer immediately after harvest and used for cytokinin analysis.

Cytokinin isolation

The samples were ground with mixture machine and added with 500 ml of 80% ethanol and kept for 12 h in the refrigerator. After 12 h, samples were filtrated and washed the residue with 500 ml of 80% ethanol and keep for 12 h. The same procedure was repeated 3 times. Total 1500 ml of 80% ethanol extractant was collected after 48 h. Extraction was concentrated in vacuum at 35 ^{0}C by using a rotary evaporator and made it to the volume of 100 ml. The P^H was adjusted to 2.5 with 1 N HCl. PVP 5 g was added to the sample and filtrated. The 30 ml Ethyl acetate was added to the sample. The samples were filtrated with

Dowex 50 W X 4 (Mesh) and washed with 100 ml of 80% ethanol. The samples were diluted with 200 ml 5N NH_4OH and cytokinin were separated. Finally, the samples were dried in vacuum by Rotary evaporator and 5 ml of 35% ethanol was added to samples and kept in the freezer. Cytokinin samples were purified by using Column Chromatography SephadexLH_2O (2.5 cm x 90 cm) and impurities were separated. The 40 ml of cytokinin samples were taken and repeated 4 times. Samples were dried in vacuum by Rotary evaporator and mixed with 5 ml water and standards of kinetin were prepared and cytokinin concentration was 0.5 ml mixed in the media.

Soybean callus bioassay by culture in vitro

Culture media were prepared by using Miller (1967) Media. First of all macro and micronutrients were measured and put into the cylinder. The α- NAA and kinetin were put into the cylinder and poured macro and micronutrient solutions. Then vitamins were added. The media were adjusted with KOH to 5.8 prior to use. All solutions were heated for 30 min. with electric heater and stirred. They were autoclaved at 15 lb/in at 121^oC for 15 min.

Soybean seeds were collected and washed with tap water followed by rinsing with distilled water in the clean bench. Soybean seeds were surface sterilized for one min. with 70% ethanol, soaked in 1% sodium hypochlorite plus a drop of liquid detergent. Then seeds were shaken gently for 20 min. followed by 10 times rinse with distilled water. Seeds were put into culture tube containing 10 ml Miller medium. Culture tubes were placed in a growth chamber under the following conditions: 27^oC, in darkness, RH 70-80%. After 15 days calli were grown.

Culture media were prepared 2nd time as described above. In this step, extracted plant cytokinin from different treatments was used as growth regulator and kinetin was used as standard. Grown calli were cut into different pieces and cultured in different replication of treatments and standard of kinetin. The calli were weighed after 2 weeks of growth. This weight showed the grade of cytokinin content in different treatments.

Design and Statistical analysis

Treatments were set following completely randomized design repeated in different trees. Mean separation was done by Least Significant Difference Test (LSDT) at 5 % level of significance.

RESULTS AND DISCUSSION

Expt. 1

Table 1 shows the effect of partial and complete phloem cut ringing on phloem tissue growth after treatment application. Phloem tissue growth was higher in the PPCR and control than CPCR treated trees (Table 1). Free ABA content was higher in PPCR and CPCR than control trees (Table 2). In CPCR trees, free ABA content exhibited higher than PPCR and control trees. A similar trend was found for Bound ABA content in complete ringing trees. In PPCR trees, free ABA content showed higher than PPCR and control trees. Total ABA content was the highest in PPCR and CPCR than control (no phloem cut ring) trees (Table 2). Fig. 1. shows phloem tissue ringing structure as affected by partial and complete phloem cut (PPCR and CPCR). Fig. 2. shows the methylation process with diazomethane for the ABA samples from peach leaves.

Expt. 2

Cytokinin content represented by callus weight was higher in control trees than partial and complete phloem cut ringing (Table 3). There was a significant difference of cytokinin represented by callus weight between complete phloem cut ringing (CPPR) and partial phloem cut ringing (PPCR) as well as control (no phloem cut). The lowest callus weight was found in complete phloem cut ringing (CPPR) trees.

The results showed that phloem tissue growth was higher in the control (no phloem cut) and PPCR than in CPCR treated trees. CPCR and PPCR are effective to regenerate more phloem tissue as dwarfing technique in young peach trees by stress phenomenon as a result of producing more ABA content after blocking translocation of photosynthesis from leaves to roots (Hossain and Mizutani, 2009). Arakawa *et al.* [4] also reported similar result. They mentioned that stem growth was significantly increased in apple trees due to the bark tissue stress. Onguso *et al.* [5] reported similar result. They reported that sugar and starch contents were higher in the CPCR and PPCR than in control. The phloem cut decreased starch content in root system and accumulated of sucrose in the leaves [14, 15]. In case of PPCR and CPCR trees, sucrose accumulated in the leaves and caused stomata closure resulting ABA increased and cytokinin decreased in leaves, shoots and twigs then it became growth inhibitory effect (14, 15). They also reported that ABA accumulated in leaves and cause the stomata to close, reducing transpiration and preventing further water loss.

Hossain *et al*. (2006, 2007) reported similar result. They stated that trunk tissue circumference was higher above the ring than below the ring in peach trees (Onguso *et al.* 2004). Mullins (1967) stated that cytokinin successfully stimulated for root growth of young grapes. Antognozzi *et al*. (1993) reported that cytokinin activating compound N_1-(2-chloro-4pyridyl)-N_3-phenylurea (CPPU) increased the transverse diameter, size and fresh weight of olives. Park *et al*. (1997) observed that stem growth of kinetin treated persimmon trees was higher than control trees. Cytokinin and other plant growth hormones stimulated cell division (cytokinesis) and influenced the pathway of differentiation by stimulating RNA and protein synthesis (Khamis and Holubowicz, 1978). Hossain (2006) found that ABA was higher in PPCR and CPCR trees than in control. They reported that it might be due to the more stress (partial phloem cut) or blocking (complete phloem cut) of the translocation of photosynthetic products (sugar and starch) from leaves to roots tissues.

CONCLUSION

Our results conclude that and CPCR and PPCR produced more ABA hormones in PPCR and CCPR than control (no phloem cut). However, CPCR and PPCR produced less cytokinin content represented by callus culture than control (no phloem cut).

ACKNOWLEDGMENTS

The authors are grateful to the Ministry of Education, Culture, Sports, Science and Technology, Japan for providing fund in this research and also Ph.D. and MS students to for their technical assistance.

REFERENCES

1. Arakawa O., K. Kanno, A. Kanetsuka and Y. Shiozaki, (1997), Effect of girdling and bark inversion on tree growth and fruit quality of apple, *Acta Hort*., 45, 579-586.
2. Antognozzi, E., Proietti, P. and Boco, M. (1993). Effect of CPPU (cytokinin) on table olive cultivars. Acta Hort. 329: 153-155.
3. Goldschmidt, E. E., R. Goren, Z. E. Chen and S. Bittner, (1973). "Increase in Free and bound Abscisic acid during neutral and ethylene induced senescence of citrus fruit peel" *Plant Physiol.*, 51, 879-882.
4. Gonzalo R.M. (2014). Abscisic acid against cancer. Patent No. US8389024 B2.
5. Hossain, ABMS, F. Mizutani, J.M. Onguso, A.R., AlShereif, H. Yamada. (2007) Inhibiting peach-trees growth with Abscisic acid, hinokitiol, and tropolone applied to partially ringed bark strips. Journal of the Horticultural Science and Biotechnology, 82:175-178.
6. Hossain, ABMS, F. Mizutani. (2009) Growth regulatory effect of cytokinin in peach trees as affected by ringed bark strip by using bioassay in vitro. Canadian Journal of Pure and Applied Sciences. 3 (1), 655-659.

7. Hossain, ABMS, Musamm M. Uddin. 2017. "Abscisic acid (ABA) hormone causes plant and tissue growth inhibition: Prospect for cancer cell growth inhibition", *International Journal of Biotech Trends and Technology (IJBTT)*, V7(4): 18-21.
8. Jose, A. (1997) "Effect of girdling treatments on flowering and production of mango" *Acta Hort.*, 1997, 455,132-134.
9. Livingston V.W.C, (1984). The conquest of cancer: Vaccines and diet. Franklinwatts, New York.NY.
10. Kieber JJ (March 2002). "Tribute to Folke Skoog: Recent Advances in our Understanding of Cytokinin Biology". J. Plant Growth Regul. **21** (1): 1–2. doi:10.1007/s003440010059. PMID 11981613.
11. Khamis, M. and T. Holubowicz, "Effect of foliar application of alar, ethrel, ABA and CCC on the frost resistance of peach trees", *Acta Hort.*, 1978, 81, 67-72.
12. Miller, C. (1967). Cytokinins in Zea mays. Ann NY Acad Sci. 14: 251-257.
13. Mullins, MG. (1967). Morphogenetic effects of roots and some synthetic cytokinins in Vitis vinifera L. J. Exptl. Bot. 18: 206-214.
14. Mauseth, J. D. (1991). Botany: An Introduction to Plant Biology. Philadelphia: Saunders. pp. 348-415.
15. Onguso, J. M., F. Mizutani and A. B. M. S. Hossain, (2004). "Effects of partial ringing and heating of trunk on shoot growth and fruit quality of peach trees" *Bot. Bull. Acad. Sin.*, 2004, 45, 301-306.
16. Park, Mi-Jin., B. Kim and S. Kang, (1997). "Effect of kinetin and B9 on the growth and carbohydrate partitioning in one –year-old trees of persimmon", Proc. 1st Int. Persimmon Symposium. Acta Hort., 436, 365-373.
17. Raven, P. H., Evert, R. F., and Eichhorn, S. E. (1992). Biology of Plants. New York: Worth. pp. 545-572.
18. Salisbury, F. B., and Ross, C. W. (1992). Plant Physiology. Belmont, CA: Wadsworth. pp. 357-407, 531-548.
19. Wright, S. T. C., and R. W. P. Hiron. (1969), "Abscisic acid, the growth inhibitor induced in detached leaves and by a period of wilting", *Nature*, 224, 719-720.
20. Zhao HW et al. (2007). Effect of induction of differentiation of TCA813 cells affected by Abscisic acid in vitro. Hua Xi Yi Xue Za Zhi 25: 508-512.

Table 1. Phloem tissue growth of peach trees as affected by partial ringing and complete ringing (PR and CR). Mean in column followed by the same letter is not statistically different at 5 % level of significance by Least significance difference test (LSDT).

Treatment	Phloem tissue growth (%)	
	Initial	
Control (no ring)	0	11.1a
Partial ringing (PR)	0	13.2a
Complete ringing (CR)	0	6.1 b

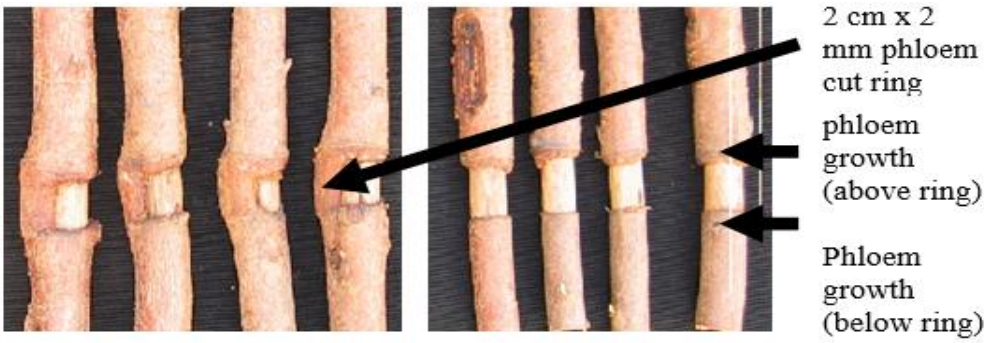

Fig. 1. Photo shows phloem tissue ringing structure as affected by partial and complete phloem cut (PPCR and CPCR).

Table 2. Free, bound and total ABA contents in leaves of peach trees as affected by partial and complete ringing (PR and CR). SE (n = 4). Mean in column followed by the same letter is not statistically different at 5 % level of significance by Least significance difference test (LSDT).

Treatments	Free ABA (ng/fw)	Bound ABA (ng/fw)	Total ABA (ng/fw)	
Control	0.6c	1.7c	2.3c	
Partial phloem cut	3.2b	4.0b	7.2b	
Complete phloem cut	5.1a	7.6a	11.7a	

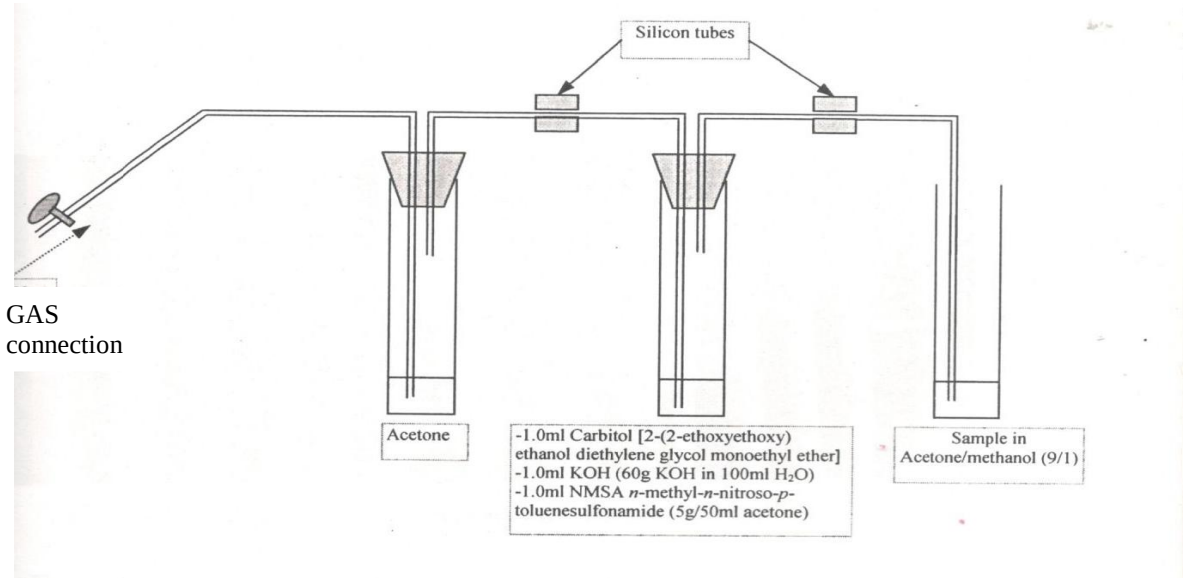

Fig. 2. Diagram showing the methylation process with diazomethane of the samples of peach leaves. The tubes arrangement and reagents used for the methylation of Abscissic acid (ABA).

Table 3. Callus weight affected by cytokinin in different treatments. Bars represented SE (n = 4). Mean in column followed by the same letter is not statistically significant at the 5 % level by LSD test.

Treatments	Callus weight (mg/gfw)
Control	0.31a
Partial phloem cut	0.28a
Complete phloem cut	0.19b