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Influence of gibberellic acid and arbuscular mycorrhizae inoculation on carbon metabolism, growth, and diterpene accumulation in *Taxus wallichiana* Zuccarini var. mairei

A. Misra*, N. K. Srivastava, A. K. Srivastava and S. K. Chattopadhyay

Central Institute of Medicinal and Aromatic Plants, P. O. CIMAP, Lucknow-226015, India.

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Changes in growth parameters and $^{14}C0_2$ and [U-^{14}C]-sucrose incorporation into the primary metabolic pools and diterpene 10 DAB compound were investigated in leaves and stems of *Taxus wallichiana* Zucarnii treated with gibberellic acid (GA) and inoculated with arbuscular mycorrhizae (AM). Compared to the control, GA(1000 ppm) and AM (1 kg/ha each) with AM-GA combined treatments, induced significant phenotypic changes and a decrease in chlorophyll content, CO_2 exchange rate and stomatal conductance. Treatment with AM-GA led to increased total incorporation of CO_2 into the leaves whereas total incorporation from ^{14}C sucrose was decreased. When $^{14}CO_2$ was fed, the incorporation into the ethanol soluble fraction, sugars, organic acids, and essential oil was significantly higher in AM-GA treated leaves than in the control. However, [U-^{14}C] -sucrose feeding led to decreased label incorporation in the ethanol-soluble fraction, sugars, organic acids, and diterpenes compared to the control. When $^{14}C0_2$ was fed to AM-GA treated leaves, label incorporation in ethanol-insoluble fraction, sugars, and oils was significantly higher than in the control. In contrast, when [U-^{14}C]-sucrose was fed the incorporation in the ethanol soluble fraction, sugars, organic acids, and oil was significantly lower than in the control. Hence the hormone treatment induces a differential utilization of precursors for oil biosynthesis and accumulation and differences in partitioning of label between leaf and stem. GA and GA-VAM influence the partitioning of primary photosynthetic metabolites and thus modify plant growth and 10-DAB compound accumulation.

Key words: Amino acids, chlorophyll, CO_2- and C-sucrose incorporation, organic acids, primary photosynthetic metabolites, stem, stomatal conductance, sugars, transpiration rate.

INTRODUCTION

Taxus wallichiana Zuccarini var. mairei is one of the main sources of diterpene 10 DAB Compound taxoids, which are used widely in, pharmaceutical industry (Suffnes, 1995). Diterpene biosynthesis in Himalayan Yew (*T. wallichiana* Zucarnii) including other monoterpenes bearing plant is strongly influenced by several intrinsic and extrinsic factors (Lawrence, 1986; Bernard et al., 1990) including temperature (Clark and Menary, 1980a), photoperiod (Burbott and Loomis, 1967), photosynthetic photon flux density (Clark and Menary, 1980b), nutrition (Srivastava and Luthra, 1994, Srivastava et al., 1997),genotype (Srivastava and Luthra, 1991), ontogeny (Srivastava and Luthra, 1991b), and osmotic stress (Charles et al., 1990). Diterpenes especially Taxoids, composed mainly of Paclitaxol (Taxol^{R}) which is mainly occurring naturally as highly taxane diterpene amides are synthesized through the mevolanate-isoprenoid pathway in the epidermal oil glands which are carbon-heterotrophic and hence depend on the adjoining mesophyll cells for precursors (McGarvey and Croteau, 1995). However, these diterpenes may not only be accumulated but also biosynthesized in leaf mesophyll cells (Gershenzon et al., 1989). Among precursors, CO_2 and sucrose are preferred for monoterpenes and diterpene

*Corresponding author. E-mail: amisracimap@yahoo.co.in. Fax: 91-0522-342666.

Abbreviations: Chl, chlorophyll; ***E*,** transpiration rate; ***g*$_s$,** stomatal conductance; /**''N,** net photosynthetic rate.

biosynthesis (Gershenzon and Croteau, 1991, 1993). Diterpene and monoterpene biogenesis is also linked to the contents of primary metabolites (Srivastava and Luthra, 1991a), and a positive but insignificant association has been shown with net photosynthetic rate, P_N (Srivastava et al., 1990). Thus the secondary metabolic pathway is closely associated and dependent on the primary metabolic pathway. Taxoids are produced in the leaf needles. Most paclitaxol taxoids produced via semisynthetic conversion of 10 - DAB (10-deacetylbaccatin III). Paclitaxol is a naturally occurring taxane diterpene amide that has been proven effective in treating various types of cancer such as ovarian carcinoma, metastatic breast cancer, non small cel cancers, adinocarcinoma and squmous cell carcinoma of the oesophagus (Morita et al., 2005).

Growth hormones play a dominant role in the regulation of growth and development by affecting sink-source relationship (Marschner, 1986). El-Keltawi and Croteau (1986a, b) reported the influence of phosphon-D, cycocel, ethephon, and daminozide on the constituents of essential monoterpene oil(s) of *M. piperita.* Farooqi and Sharma (1988) reported influence of growth retardants on growth and essential oil accumulation in *M. arvensis* whereas Srivastava and Sharma (1991) reported the influence of triacontanol on photosynthetic characteristics and oil accumulation in *M. arvensis.* Most of the growth hormone studies on monoterpene bearing plants attribute the effects to the influence on enzymes of biosynthetic pathways and on plant and growth characters such as herb yield and leaf/stem ratio. However, it is not clear what changes occur in the photosynthetic C-metabolism of the hormone treated plant and translocation of assimilates to the Diterpene accumulation. While studying the influence of growth hormones on yield and growth, we observed significant and persistent effect of GA and GA-AM on plant phenotype. *T. wallichiana* Zucarnii (Himalayan Yew) is a very slow growing high altitude forest tree. Hence, this hormone treatment alone and in combination with AM study on Himalayan Yew has been taken for rapid growth and carbon metabolism on the photosynthesis and photosynthatates effect on the physiology of the plant.

Further, in the present study we report the influence of GA and GA-AM on the photosynthetic efficiency and diterpene 10-DAB paclitaxol accumulation studies during the incorporation of $^{l4}C0_2$ and [U-^{,4}C]-sucrose into primary photosynthetic metabolites, sugars, amino acids, and organic acids, and simultaneously into the taxol bearing compounds of *T. wallichiana* plants seedlings brought from high altitude CIMAP field station Purara, Almora treated plants. Changes in P_N, chlorophyll (Chi) content, and stomatal conductance (g_s) were also determined.

MATERIALS AND METHODS

Uniform suckers of *M. spicata* cv. MSS-5 obtained from the farm nursery of the Institute were treated with GA and GA-VAM (1 kg nr^3 each) by dipping in respective solution for 24 h. Later the treated suckers were planted in 10 000 cm^3 earthen pots maintained in a glasshouse at ambient temperature (30 - 35°C) and irradiance (800 - 1000) |amol rtr^{2}s^{-1}, measured by a *LiCOR* light meter model *188 B).* Values of growth characters, essential oil, and tracer feeding were recorded 100 d after the treatment.

Chl *(a + b)* content was measured on the third brances of the needle like leaf. A known mass of leaf tissue was extracted with 80% acetone and the absorbance was recorded by a *Milton Roy* spectrophotometer *Spectronic 21 D* using the method of Arnon (1949). P_N, initial transpiration rate (£), and g_s of the third leaf were measured in a closed system using a portable computerized photosynthesis model *Li-6000 {LiCOR,* Lincoln, USA) as described in Srivastava and Luthra (1991a). For determining the extraction of taxoids from the control plants (untreated) or after feeding of $^{14}CO_2$ or [U-^{14}C]-sucrose, a known mass of shoot (leaf + stem) material was finely chopped and subjected to percolate in pure methanol at room temperatre as described by earlier (Chattopadhyay and Sharma, 1995). The radioactivity in ether samples was determined in a scintillation counter *(LKB Rack Beta 1215)* using a PPO-POPOP-toluene cocktail (Srivastava and Luthra 1991a).

The tracer studies were carried out with $^{14}CO_2$ and [U-^{14}C]-sucrose that were fed to the freshly excised shoots of treated and control plants and the amounts of label incorporated into 10-DAB and simultaneously into the pool of primary photosynthetic metabolites (sugars and sugar phosphates, amino acids, and organic acids) were determined. Before the labelling studies, the shoots were cut under water and tested to ensure that they were able to take up water properly. For $^{14}CO_2$ studies, 12 unbranched main shoots (of GA-AM treated, GA treated, and control plants) having 6 leaf pairs were placed in vials with the cut ends dipped in half strength Hoagland and Arnon (1938) solution. The vials were then placed in a sealed plexiglass chamber (20 000 cm^3 capacity) around a central vial containing $Na_2{}^{14}C0_3$ solution (3.7 MBq, 2.8 GBq mmol^{-1}) obtained from the isotope division of *Bhabha Atomic Research Centre* (BARC), Trombay, India. $^{14}CO_2$ was generated by injecting 4 N H_2SO_4 into carbonate solution through a PVC tube and uniformly distributed with the help of a small electric fan. The leaves were allowed to assimilate $^{14}C0_2$ for 1 h in sunlight (800 - 1000 |amol m"^{2} s"^{1}). At the end of 1 h, a saturated solution of KOH was run into the central vial and left for 15 min to absorb excess $^{14}CO_2$. The chamber was then opened for the remaining incorporation period of 6 h (Srivastava and Luthra 1991a). For feeding experiments with [U-^{14}C] -sucrose, unbranched shoots having 6 leaf pairs each were placed in vials containing an aqueous solution of 1 |amol [U-^{14}C]-sucrose (185 kBq) obtained from the Isotope Division of BARC, Trombay, India (specific activity 21.5 GBq mmol"). After the uptake of labelled material, the vials were kept filled with half strength Hoagland solution, and the samples were harvested after 6 h.

After exposure to $^{14}CO_2$ or [U-^{14}C]-sucrose feeding, plants were separated into leaf and stem, finely chopped, and divided into two parts:

1.) A known weight of leaf, stem and root tissues were processed for determining the incorporation of current photosynthetic metabolite in total diterpenes. The radioactivity in alkaloid fraction was determined using PPO-POPOP-Toluene cocktail in a liquid scintillation counter. The unit of expression was Bq/g.dry wt. of tissue (leaf, stem and roots).

2.) A known weight of leaf, stem and root tissues were immediately fixed into boiling ethanol so that the current metabolic status was maintained .The plant material was ground in ethanol, filtered, filtrate evaporated and diluted in a known volume of aqueous phase; termed as ES fraction. This aqueous phase was further extracted with chloroform and this CS fraction contained pigments

Figure 1. Changes in plant characters *of Taxus wallichiana* due to hormone and hormone-AM treatment. Left (A): Control:, middle: (B): AM-GA, and right: (C): GA.

and some of the terpenoid pathway derived end metabolites. The remaining plant material termed, as EIS fraction was further hydrolyzed by enzyme diastase in 0.05 M acetate buffer (pH 5.2) at 50℃ (Srivastava et al. 1990). The label in ^{14}C in ES and in EIS fraction was determined in Bray's scintillation fluid and in CS fraction in PPO-POPOP-Toluene cocktail in a liquid scintillation counter. The unit of expression was Bq/g.fresh wt. of tissue (leaf, stem and roots). Total ^{14}C incorporated was expressed as sum of values of ES+EIS+CS fraction. The ES fraction was further separated into metabolic pool consisting of neutral (sugar+sugar phosphates) acidic (organic acids) and basic (amino acids) fractions by separation through Amberlite ion exchange column chromatography. The ^{14}C content in eluates after column chromatgraphy was determined in Bray's scintillation fluid in a liquid scintillation counter (Srivastava and Luthra 1991b), for determining the incorporation of label into taxoids 10-DAB a known mass of shoot material (leaf + stem) was processed as described earlier (Chattopadhyay and Sharma, 1995). Further, for determining incorporation of label into primary photosynthetic metabolites a known mass of tracer-fed leaves and stem was extracted immediately in boiling 80% ethanol. The stem sample did not include the basal portion which had been immersed in labelled sucrose. The ethanol soluble material was separated into neutral (sugars and sugar phosphates), basic (amino acids), and acidic (organic acids) fractions by *Amberlite* ion exchange column chromatography. Ethanol-insoluble material was hydrolyzed by diastase in 0.05 M acetate buffer (pH 5.2) at 50℃. The radioactivity in hydrolyzed alcohol insoluble material and in eluates after ion exchange separation was measured using Bray's scintillator (Srivastava and Luthra 1994). Total ^{14}C incorporated was calculated as the sum of the total label incorporated in ethanol-soluble and -insoluble fraction and expressed on fresh mass basis.

All measurements were taken in triplicate and the results are given as means ± SE. Values were statistically analysed for significance by paired /-test.

RESULTS AND DISCUSSION

Treatments with GA and GA-AM significantly increased plant phenotype which was evident even at 100 d of growth (Figure 1). Normally the plant metabolizes the externally applied hormones and even if there are some phenotypic differences, these are temporary and the plant reverts soon to its normal phenotype, but in the present case the hormone effects were evident much longer. This was accompanied by marked changes in physiological characteristics.

The GA treated plants had significantly lower contents of ChL *(a+b),* Chi *a,* increased P_N, g_s, *E,* and plant height as compared to control (Table 1). Thus the overall growth was in increased. There was a difference in utilization pattern of $^{14}CO_2$ and [U-^{14}C] -sucrose. When $^{14}C02$ was fed, the total $^{14}CO_2$ fixed in leaves in GA treatment was significantly higher than in the control. Also the ethanol-insoluble fraction, the sugars, organic acids, and essential oil had a significantly higher ^{14}C-incorporation in GA treated leaves than in the control (Table 2). Thus the GA-AM treated plants allocated more photosynthetic metabolites towards diterpene taxoids than the control plants. Partitioning of photosynthetic metabolites between leaf and stem is an important factor in yield determination (Srivastava and Luthra 1991a). In stems, ^{14}C incorporation in ethanol soluble fraction, sugars, and organic acids was significantly higher in the GA-AM treated plants than in the control. Thus, ethanol soluble compounds remained untranslocated in the stem (Table 2). Overall, if we will go for phenotypic changes the plants in Figure 1 showed the more increase in height then the control one. Generally, the increase in height is only3 inches in a year where as this height increase is only in 4 months.

When [U-^{l4}C]-sucrose was fed to GA treated leaves, the total ^{14}C incorporation was significantly higher than in the control. Incorporation into ethanol soluble fraction was significantly higher than that measured in the

Table 1. Changes in growth and yield characters of *T. wallichiana* treated with etherel and gibberellic acid (GA). Chi = chlorophyll; *P^* = net photosynthetic rate.

Characters	GA	Control	GA-AM
Chl *a* [g kg-'(FM)]	2.19 ± 0.06*	2.72 ± 0.21	1.79 ± 0.03*
Chl b[gkg-'(FM)]	0.74 ± 0.08*	1.15 ± 0.08	0.55 ± 0.03*
Chl *(a+b)* [g kg-'(FM)]	2..93 ± 0.05 *	3.87 ± 0.27	1.34 ± 0.01*
P_N [ng($C0_2$) m^{-2} $s"^1$]	164 ± 7*	129 ± 4	134 ± 12*
Initial transpiration rate [mmol nr^2 $s"^1$]	476 ± 30*	691 ± 90	446 ± 20*
Stomatal conductance [mmol m^{-2} s-']	229 ± 11*	429 ± 10	271 ± 20*
Plant height [cm]	95 ± 0.21**	61 ± 0.10	81.85 ± 0.05**

*/** Mean values significant at 5/1% level of significance by pair r-test; NS – non significant.

Table 2. Changes in incorporation pattern of $^{14}CO_2$ into primary photosynthetic metabolic pool and diterpene: taxoids in leaves and stems *of T. wallichiana* treated with GA-AM and gibberellic acid (GA).

	Fractions	GA-AM	Control	GA
Leaves	Ethanol-soluble fraction	238 ±16^{NS}	282 ± 27	2691 ± 5^{NS}
	Ethanol-insoluble fraction	1749 ±13**	1256 ±53	1818 ± 49*
	Total incorporation	1917 ±43**	1301 ± 57	4395 ± 41*
	Sugar	108 ±6**	49 ± 2	75 ±1*
	Amino acids	317 ±2^{NS}	171 ± 72	747 ± 83^{NS}
	Organic acids	127 ±5**	77 ± 2	111 ± 2^{NS}
	Taxoids 10-DAB	1.41 ± 0.02**	0.42 ± 0.01	1.31 ± 0.02*
Stem	Ethanol-soluble fraction	147 ± 6*	79 ± 2	382 ± 23*
	Ethanol-insoluble fraction	1301 ± 71^{NS}	401 ± 07	2791 ± 47"
	Total incorporation	1577 ± 71^{NS}	523 ±19	3298 ±301**
	Sugar	127 ±25*	49 ± 3	189 ± 27^{NS}
	Amino acids	901 ± 103^{NS}	148 ± 33	1491 ± 329^{NS}
	Organic acids	154 ± 7*	39 ± 3	186 ± 17*

All values in 10^3 dps kg'"(FM). */** Mean values significant at 5/1% level of significance by pair test; NS – non significant.

insoluble fraction. However, the label in sugars, organic acids, and essential oil fraction was significantly lower than in the control (Table 3). When these fractions were analyzed in the stem, the ethanol-insoluble fraction had significantly higher label whereas the ethanol-soluble fraction had significantly lower amounts of labeled sugars, amino acids, and organic acids than the controls (Table 3). Thus, the amount of compounds derived from added [U-^{14}C]-sucrose was higher in leaves and was significantly lower in stems in GA treated plants. Hence the capacity to utilize end products of photosynthetically fixed $^{14}CO_2$ and the externally applied sucrose was entirely different. Ontogenic changes exist for distribution of photosynthetically fixed $^{14}C0_2$ in peppermint leaves. The incorporation of $^{14}CO_2$ into sugars was maximum followed by organic acids, amino acids, and essential oil at all stages of leaf development. The incorporation into sugars and amino acids declined as the leaf matured whereas the incorporation into monoterpenes and organic acids increased with leaf expansion and then decreased (Srivastava and Luthra 1991b). In onions, the older was the plant the more of C-assimilate left the source leaf (Khan, 1981).

The GA-AM treated plants had significantly lower contents of ChL pigments, P_N, *E,* and g_s, however the plant height was significantly higher than in the control (Table 1). GA-AM treatment resulted in both higher total fixation of $^{14}CO_2$ and ^{14}C incorporation in ethanol-insoluble fraction and sugars of leaves. Significantly higher amounts of photosynthetic metabolites were translocated towards essential oils because the label was significantly higher in essential oil (Table 2). Amino acid and organic acid contents were not significantly affected over control. Similarly, the stem of GA-AM treated plants showed significantly higher total incorporation, contents of ethanol-soluble and -insoluble fraction, whereas the contents of organic acids, amino acids, and sugars were not significantly different than in the control (Table 2).

Table 3. Changes in incorporation pattern of [U- C]-sucrose into primary photosynthetic metabolites and in diterpene: taxoids in *T. wallichiana* treated with GA-AM and gibberellic acid (GA).

	Fractions	GA-AM	Control	GA
Leaves	Ethanol-soluble fraction	6113 ± 27"	3571 ± 731	1429 ± 323*
	Ethanol-insoluble fraction	2258 ± 148^{NS}	3192 ± 321	4321 ± 1108^{NS}
	Total incorporation	9714 ± 139"	5839 ± 1407	2142 ± 1408"
	Sugar	694 ± 92"	2831 ± 220	1955 ± 87^{NS}
	Amino acids	549 ± 12^{NS}	76 ± 4	148 ± 3*
	Organic acids	53 ± 7*	137 ± 3	128 ± 4*
	Taxoids 10-DAB	1.03 ± 0.04**	1.19 ± 0.07	9451 ± 170**
Stem				
	Ethanol-soluble fraction	10549 ± I489*	12201 ± I158	I4629 ± 325*
	Ethanol-insoluble fraction	3754 ± 133*	1261 ± 299	2512 ± 377^{NS}
	Total incorporation	14534 ± 1613^{NS}	14521 ± 1234	18251 ± 212^{NS}
	Sugar	823 ± 47	1128*49	I191 ± 11^{NS}
	Amino acids	61 ± 1*	72 ± 4	98 ± 3^{NS}
	Organic acids	88 ± 2*	116 ± 3 *	98 ± 3*

All values in 10^3 dps kg"'(FM). */** Mean values significant at 5/1% level of significance by pair test; NS – non significant.

Thus overall incorporation of $^{14}CO_2$ into metabolites and their higher subsequent translocation to oil biosynthetic pathway were higher in GA-AM treated plants. Here, the GA-AM treated plants showed the increase in height and the increase in number of branches in comparison with the control (Figure 1).

As far as the utilization pattern of [U-^{14}C]-sucrose is concerned, GA-AM treatment resulted in leaves in significantly higher total incorporation, incorporation in ethanol-soluble fraction, amino acids, and essential oil, whereas ethanol-insoluble fraction and sugar contents were not significantly influenced (Table 3). In contrast, the contents of all these metabolites in stem were significantly not affected (Table 3).

Application of GA significantly increases growth in terms of height and physiological parameters which negatively affects herb yield. Hormone application in general does not bring a simultaneous increase in growth and diterpenes. In Japanese mint, chlormequat chloride increased monoterpene content but inhibited growth whereas ethephon decreased growth but had no significant effect on monoterpene content (Farooqi and Sharm, 1988). Hormones such as phosphon-D and daminozide influence enzymes and interconversion in monoterpene biosynthesis (El-Keltawi and Croteau 1996a, b) and endogenous content of other hormones. However, it is not known how the carbon fixation capacity is affected by hormone application. Despite the decrease in herb yield, both hormone-treated plants contained higher amounts of the $^{14}CO_2$ fixation products. This probably results in greater translocation of photosynthetic metabolites to the oil biosynthetic pathway. The higher contents do not necessarily mean higher $^{14}CO_2$ efficiency; it could also mean that the fixed $^{14}CO_2$ is not utilized by the plant growth process whereas in control plants it is utilized and its content is lower.

The fed sucrose was poorly utilized for oil biosynthesis and simultaneously the content of photosynthetic metabolites was also low. Thus the utilization of $^{14}CO_2$ and sucrose for oil biosynthesis was different. The changes in growth could also be due to differences in partitioning of available assimilates between leaf and stem. The monoterpene/diterpene biosynthesis is an integration of several metabolic pathways which require linking of several steps such as continuous production of precursors, their transport and translocation to the active site of synthesis, and finally the diterpene taxoids 10-DAB. This sequence of steps depends on normal functioning of associated metabolic pathways. Any disruption in normal metabolic pathways affects the sequence of steps in oil biosynthesis. Thus a plant may alter/adopt its metabolic pathway in response to particular effect, such as nutrient imbalance especially the P and Zn, hormone application, *etc.* Under GA and GA-AM treatment there is higher accumulation of photosynthetic metabolites, nevertheless, the decrease in herb yield and growth may be due to energy deficiency, membrane effects, or other control mechanisms which need to be investigated. Further the increase in height due to the application of GA, GA-AM visualized the rapid elongation in the slow growing Himalayan Yew.

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