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Full Length Research Paper

Influence of etherel and gibberellic acid on carbon metabolism, growth, and alkaloids accumulation in *Catharanthus roseus* L.

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Changes in growth parameters, $14C0_2$ and [U-14C] -sucrose incorporation into the primary metabolic pools and essential oil were investigated in leaves and stems of *Catharanthus roseus* L. treated with etherel and gibberellic acid (GA). Compared to the control, GA and etherel treatments induced significant phenotypic changes and a decrease in chlorophyll content, carbon assimilation rate, and stomatal conductance. Treatment with etherel led to increased total incorporation of CO_2 into the leaves where as total incorporation from 14C sucrose was decreased. When $14CO_2$ was fed, the incorporation into the ethanol soluble fraction, sugars, organic acids, and essential oil was significantly higher in etherel treated leaves than in the control. However, [U-14C] -sucrose feeding led to decreased label incorporation in the ethanol-soluble fraction, sugars, organic acids, and essential oils compared to the control. When $14C0_2$ was fed to GA treated leaves, label incorporation in ethanol-insoluble fraction, sugars, and oils was significantly higher than in the control. In contrast, when [U-14C]-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. Etherel and GA influence the partitioning of primary photosynthetic metabolites and thus modify plant growth and alkaloid accumulation.

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

INTRODUCTION

Catharanthus roseus L. (Periwinkle) is one of the main sources of alkaloids, mainly catharanhine, vindoline, ajmalcine, serpentine, vinblastine and vincristine used widely in pharmaceutical industries for the preparation of diabetic drugs, cardiac drugs, in hypertension and in anti-cancerous drugs formation. Thus overall a miracle plant which is having the alkaloids biomolecules of enourmous usage in pharmaceutical sciences. Alkaloids biosynthesis in periwinkle including *C. roseus* L. *Cv. Dhawal* of white flowered one and *C. roseus* L. *Cv. Nirmal* of pink flowered one is strongly influenced by several intrinsic and extrinsic factors (Lawrence, 1986; Bernard et al., 1990) including temperature (Clark and Menary, 1980b), photoperiod (Burbott and Loomis, 1967), photosynthetic photon flux density (Clark and Menary, 1980a), 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). Alkaloids, composed mainly of terpene indole alkaloids (TIA's), is synthesized through the mevolanate-isoprenoid pathway in the epidermal cells, which are carbon-heterotrophic and hence depend on the adjoining mesophyll cells for precursors (McGarvey and Croteau, 1995). The TIAs product accumulation occurs in different plant parts such as *vincristine* and *vinblastine* (anti cancer alkaloids: that are specific to leaves) and serpentine and ajmalicine (antihypertensive alkaloids: which are localized in roots). During the growth of the plant the pattern of total TIAs product accumulation increases from leaf to roots and distribution is highest in roots, then in leaves and lowest in stems at flowering

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in stems at flowering stage (Reda, 1978). Because of its medicinal value, high price and low content in plants extensive research has been carried out to dissect the biosynthetic mechanisms with the aim of improving TIAs product accumulation so that it is commercially viable. Majority of these studies have been carried out in cell suspension cultures, specific cell lines and in tissue cultures (Moreno et al., 1995; van der Heijden et al., 1989). In planta studies are very limited (at seedling stage) and are surprisingly ignored as compared to cell culture studies. The biosynthesis and accumulation of TIAs is dependent on several intrinsic and extrinsic factors (Verpoorte et al., 1997) and several factors regulating biosynthesis has been extensively explored (Van *der* Heijden et al., 2004, Misra et al., 1996). Among these plant growth regulators (PGRs) play a crucial role in regulating overall plant growth and metabolism. This effect is mainly brought about by altering the availability and distribution of metabolites in source sink relationship within plants *(*Marschner, 1986). Thus hormonal environment must significantly influence *TIAs* product accumulation also. Despite the knowledge that biosynthesis of TIAs is under developmental control *in planta* studies have been largely been ignored, little work has been done to understand the relationship between metabolite accumulation and relationship between growth hormones and TIAs accumulation in intact plants (Verpoorte et al., 1997). Application of *PGRs* influences metabolic processes leading to the availability of precursors and/or metabolic intermediates that are subsequently utilized for TIAs product accumulation. One of the principal primary metabolic processes affected are the photosynthetic C reduction cycle in PGR treated plants, partitioning and altered distribution of primary metabolites into plant organs as leaf, stem and roots and the subsequent utilization of current photo assimilates into TIAs accumulation. However, alkaloids may not only be accumulated but also biosynthesized in leaves (Gershenzon et al., 1989). Among precursors, CO_2 and sucrose are preferred for terpene biosynthesis (Gershenzon and Croteau, 1991, 1993). Terpene 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, PN (Srivastava et al., 1990). Thus the secondary metabolic pathway is closely associated and dependent on the primary metabolic pathway.

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 monoterpenes of *M. piperita.* Farooqi and Sharma (1988) reported influence of growth retardants on growth and terpene accumulation in M. arvensis whereas Srivast*av*a and Sharma (1991) repor-ted the influence of triacontanol on photosynthetic characteristics and terpene accumulation in *M. arvensis.* Most of the growth hormone studies on *M. piperita* or *M. arvensis* 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 essential oil accumulation. While studying the influence of growth hormones on yield and growth, we observed significant and persistent effect of etherel and GA on plant phenotype. Several investigators have studied the regulatory and mechanistic role of a variety of PGRs. Methyl jasmonate (*MJa*) vapour significantly enhanced TIAs accumulation in germinating seedlings by doubling the alkaloid content and this ability to increase alkaloid content declined with age of seedlings (Aerts et al., 1994). Most of the PGR studies conducted attributed the influence of these PGRs on influence on biotransformations at the tertiary or terminal steps of the biosynthetic pathway, activity of some key enzymes as – peroxidases, strictosidine synthase, G10 hydroxylase, TDC etc. Application of PGRs influences metabolic processes leading to the availability of precursors and/or metabolic intermediates that are subsequently utilized for TIAs product accumulation. One of the principal primary metabolic processes affected are the photosynthetic C reduction cycle in PGR treated plants, partitioning and altered distribution of primary metabolites into plant organs as leaf, stem and roots and the subsequent utilization of current photo assimilates into TIAs accumulation. Many of these investigations have been targeted to understand and improve upon alkaloid in *C. roseus* cell suspension cultures. These results were not as expected for dimeric alkaloids are produced only in green shoots and productivity of other alkaloids is still too low to permit commercialization (El-Sayeed and Verpoorte, 2004). Hence understanding in planta TIAs biosynthetic accumulation is considered necessary. While studying the influence of PGRs as elicitors of TIAs accumulators in catharanthus plants we observed persistent and significant effect of GA on plant phenotype

In the present study, we report the influence of GA and etherel on the photosynthetic efficiency and alkaloid accumulation studied during the incorporation of $14C0_2$ and [U-, 4C]-sucrose into primary photosynthetic metabolites, sugars, amino acids, and organic acids, and simultaneously into the alkaloids in *C. roseus.* Changes in PN, chlorophyll (Chi) content, and stomatal conduc-tance (*gs*) were also determined.

MATERIALS AND METHODS

Uniform seedlings of *C. roseus* obtained from the farm nursery of the Institute were treated with ethereal and GA (1 kg/l) were sprayed to each seedlings) by dipping in respective solution for 24 h. Later the treated seedlings were planted in 10, 000 cm^3 earthen pots maintained in a glasshouse at ambient temperature (30-35 °C) and irradiance (800-1000 |*amol* rtr2s_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. Chi *(a+b)* content was measured on the third leaf. A known mass of leaf tis-

sue 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). PN, initial transpiration rate (£), and *gs* 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 alkaloids from the control plants (untreated) or after feeding of $14CO_2$ or [U-14C]-sucrose, a known mass of shoot (leaf + stem) material was finely chopped and subjected to the alkaloid extraction.

Growth observations

Plant height, leaf length (of the 3rd leaf that is physiologically mature) and internode's length (between 2nd and 3rd leaf) were measured. Plants were carefully uprooted and separated in leaves, stem and roots, their fresh and dry weight recorded.

Determination of chlorophyll (Chl.) content and CO2 exchange rate (CER)

A known weight of third leaf tissue was ground in cold pestle and mortar encased in ice in dim light till a fine paste was obtained. The fine paste was extracted with 80% acetone and made up to a known volume and absorbance recorded on a Spectronic 21D spectrophotometer (Milton Roy & Co., New York, USA) and chl. concentration (a, b and total) calculated according to method of Arnon (1949). CO_2 exchange rate (CER) of 3rd leaf was measured in an open system by a computerized portable photosynthesis system Model CI-310 PPS (CID Instruments, USA).

Determination of total terpene indole alkaloid content

Freshly harvested leaf, stem and root samples were oven dried at 60°C for 48 h and powdered. A known weight of each plant material was extracted in 90% ethanol, left overnight and filtered. The residue was again extracted with ethanol and pooled ethanol extract concentrated to dryness. Dried residue was redissolved in ethanol, diluted with equal volume of water and acidified with 3% hydrochloric acid. The mixture was extracted with hexane (3 times), hexane fraction discarded and aqueous extract cooled to 10°C and basified with ammonium hydroxide to pH 8.5.This extract was extracted with chloroform (3 times). The combined chloroform extract was washed with water and evaporated to dryness. The total TIA content was expressed as % dry weight of samples (Uniyal et al., 2001).

14CO2 incorporation and partitioning of assimilate into primary metabolites

Feeding experiments on intact plants were carried out 5 h after the beginning of the light period. Pots with GA treated and control plants were placed in an assimilation chamber around a central vial containing $Na14CO_3$ solution (activity 0.05 mCi, specific activity 1.78 TBq/mol) obtained from the isotope division of Bhabha Atomic Research Centre, Mumbai, India. $14CO_2$ was liberated by 2M injecting H_2SO_4 into the *carbonate* solution through a PVC inlet initially exposed to$14CO_2$ for 1h.Afterwards saturated solution of KOH was run into the central vial to absorb remaining $14CO_2$.

Natural sunlight varied between 800-1000 mol/m2/sec during the time of exposure. The chamber was then opened and the plants allowed assimilating $14CO_2$ for 6 h (Srivastava et al., 2004). After this exposure plants were gently uprooted from soil and separated into leaf, stem and root. Each of the plant part was processed for determining the distribution of label into major primary photosynthetic metabolic fractions such as ethanol soluble (ES), ethanol insoluble (EIS) and chloroform soluble (CS). Simultaneously the biosynthetic capacity to utilize currently assimilated metabolites was determined by quantifying the label in total TIAs in respective plant parts. The separated plant parts leaf, stem and roots were divided in two portions:

1. A known weight (1 g) of leaf, stem and root tissues were processed for determining the incorporation of current photosynthetic metabolite in total TIAs as explained earlier in "Determination of total terpene indole alkaloid content*"*. The radioactivity in alkaloid fraction was determined using PPO-POPOP-Toluene cocktail in a liquid scintillation counter (Wallac 1409, USA). The unit of expression was Bq/g.dry wt. of tissue (leaf, stem and roots).
2. A known weight (1 g) 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 and some of the terpenoid pathway derived end metabolites. The remaining plant material termed, as EIS fraction was further hydrolyzed by enzyme diastase in 0.05M acetate buffer (pH 5.2) at 50°C (Srivastava et al., 2004). The label in 14C 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 (Wallac 1409, USA). The unit of expression was Bq/g.fresh wt. of tissue (leaf, stem and roots). Total 14C incorporated was expressed as sum of values of ES + EIS + CS fraction. The ES frac-tion 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 14C content in eluates after column chromatography was determined in Bray's scintillation fluid in a liquid scintillation counter (Wallac 1409, USA) (Dixit and Srivastava, 2000; Srivastava and Luthra, 1994).

Statistical analysis

The results presented are mean values of three extractions and were subjected to LSD at 0.05% analysis.

RESULTS AND DISCUSION

Treatment with etherel and GA resulted in significant changes in 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 etherel-treated plants had significantly lower contents of Chi *(a+b),* Chi *a,* decreased Pn, gs, *E,* and plant height as compared to control (Table 1). Thus the overall growth was stunted. There was a difference in utilization pattern of $14CO_2$ and [U-14C] -sucrose. When $14C0_2$ was fed, the total $14CO_2$ fixed in leaves in etherel treatment was significantly higher than in the control. Also the ethanol-insoluble fraction, the sugars, organic acids,

Figure 1. Changes in plant characters of *C. roseus* due to hormone treatment. Left: ethereal, middle: gibberellic acid, right: control.

Table 1. Changes in growth and yield characters of C. *roseus* treated with etherel and gibberellic acid (GA). Chi = chlorophyll; *P^* = net photosynthetic rate. */** Mean values significant at 5/1% level of significance by pair r-test; NS – non significant.

Characters	Etherel	Control	GA
Chi *a* [g kg-'(FM)]	2.03 ± 0.07*	2.92 ± 0.26	1.09 ± 0.01*
Chl b [gkg-'(FM)]	0.84 ± 0.008NS	1.25 ± 0.12	0.45 ± 0.02*
Chi *(a+b)* [g kg-'(FM)]	2.87 ± 0.06	4.18 ± 0.37	1.54 ± 0.01*
PN [ng(C02) m-2 s"1]	114 ± 7"	229 ± 4	139 ± 12*
Initial transpiration rate [*mmol* nr2 s"1]	436 ± 30*	661 ± 90	426 ± 20*
Stomatal conductance [*mmol* m-2 s-']	224 ± 10"	440 ± 10	250 ± 20"
Plant height [cm]	3.5 ± 0.21"	6.1 ± 0.10	11.85 ± 0.05**

Table 2. Changes in incorporation pattern of l4CO2 into primary photosynthetic metabolic pool and alkaloids in leaves and stems of *C. roseus* treated with *ethere*l and *gibberelli*c acid (GA). All values in 103 *dps* kg"'(FM). */** Mean values significant at 5/1 % level of significance by pair *Mes*t; NS - nonsignificant.

Fractions	Etherel	Control	GA
Leaves ethanol-soluble fraction	225 ± 26 NS	262± 37	2667 ± 3 NS
Ethanol-insoluble fraction	1723 ± 133**	1057 ± 63	1608 ± 50"
Total incorporation	1948 ± 157**	1320 ± 97	4275 ± 48*
Sugar	128 ± 3**	60± 3	85 ± 2*
Amino acids	340 ± 7 NS	195 ± 800	703 ± 183 NS
Organic acids	140 ± 5**	83± 2	103 ± 2 NS
Alkalo	1.56 ± 0.04**	0.51 ± 0.03	1.28 ± 0.03"
Stem ethanol-soluble fraction	160 ± 8"	89± 3	345± 53*
Ethanol-insoluble fraction	1377± 717 NS	497± 17	2753 ± 377"
Total incorporation	1538± 717 NS	587± 20	3098 ± 358**
Sugar	158 ± 5*	35± 5	177 ± 47 NS
Amino acids	933 ± 283 NS	132 ± 43	1300 ± 400 NS
Organic acids	160 ± 8"	47± 3	167± 13"

and essential oil had a significantly higher, 14C-incorporation in *etherel* treated leaves than in the control (Table 2). Thus the etherel -treated plants allocated more photosynthetic metabolites towards essential oil 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, 14C incorporation in ethanol soluble fraction, sugars, and

Table 3. Changes in incorporation pattern of [U- C]-sucrose into primary photosynthetic metabolites and in alkaloids in *C. roseus* treated with etherel and gibberellic acid (GA). All values in 103 dps kg"'(FM). */** Mean values significant at 5/1% level of significance by pair Mest; NS – non-significant.

Fractions	Etherel	Control	GA
Leaves ethanol-soluble fraction	6863 ± 37"	2577± 933	15695± 583*
Ethanol-insoluble fraction	2655 ± 118 NS	3097± 350	4425 ± 1200 NS
Total incorporation	9520± 148"	5673 ± 1300	20120 ± 130O"
Sugar	665± 77"	2635± 200	1775 ± 90 NS
Amino acids	52 ± 15 NS	90± 3	113± 3*
Organic acids	53± 7*	137 ± 3	120± 5*
Alkaloids	1.01 ± 0.07**	1.93 ± 0.09	9555 ± 100**
Stem ethanol-soluble fraction	10507 ± I450*	12308 ± I267	I3637 ± 317 NS
Ethanol-insoluble fraction	3457± 125*	1757± 417	2715 ± 567 NS
Total incorporation	13963 ± 1567 NS	14067± 1667	16352 ± 267 NS
Sugar	852 ± 55	1020 ± 75	I188 ± 6 NS
Amino acids	68± 3*	82± 5	100 ± 3 NS
Organic acids	100 ± 2*	108 ± 3	108 ± 3

organic acids was significantly higher in the etherel treated plants than in the control. Thus, ethanol soluble compounds remained untranslocated in the stem (Table 2).

When [U-I4C] -sucrose was fed to etherel treated leaves, the total, 4C incorporation was significantly higher than in the control. Incorporation into ethanol soluble fraction was significantly higher than that measured in the insoluble fraction. However, the label in sugars, organic acids, and alkaloid fraction was significantly lower than in the control (Table 3). When these fractions were analysed in the stem, the ethanol-insoluble fraction had significantly higher label whereas the ethanol-soluble fraction had significantly lower amounts of labelled sugars, amino acids, and organic acids than the controls (Table 3). Thus the amount of compounds derived from added [U-14C]-sucrose was higher in leaves and significantly lower in stems in etherel treated plants. Hence the capacity to utilize end products of photosynthetically fixed CO_2 and the externally applied sucrose was entirely different. Ontogenic changes exist for distribution of photosynthetically fixed $14CO_2$ in peppermint leaves. The incorporation of $I4CO_2$ into sugars was found 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 alkalois 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-treated plants had significantly lower contents of Chi pigments, PN, *E,* and *gs,* however the plant height was significantly higher than in the control (Table 1). GA treatment resulted in both higher total fixation of $14CO_2$ and 14C 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-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). Thus overall incorporation of CO_2 into metabolites and their higher subsequent translocation to oil biosynthetic pathway were higher in GA-treated plants.

As concerns the utilization pattern of [U-14C]-sucrose, GA 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 etherel and GA significantly decreases growth and physiological parameters which negatively affects herb yield. Hormone application in general does not bring a simultaneous increase in growth and alka-loids. In Catharunthus mint, chlormequat chloride increased oil content but inhibited growth whereas ethephon decreased growth but had no significant effect on oil content (Farooqi and Sharma 1988). Hormones such as phosphon-D and daminozide influence enzymes and interconversion in essential oil biosynthesis (El-Keltawi and Croteau, 1986a; 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 CO_2 fixation products. This probably results in greater trans-location of photosynthetic metabolites to the oil biosynthetic pathway. The higher contents do not necessarily mean higher CO_2 efficiency; it could also mean that the fixed 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 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 essential oil 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 oil accumulation. 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, hormone application, etc. Under etherel and GA 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.

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