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Influence of 8-Methyl-nonenoic Acid on Capsaicin Biosynthesis in In-Vivo and In-Vitro Cell Cultures of *Capsicum* Spp.

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Capsaicin is a bioactive molecule synthesized by enzymatic (putative capsaicin synthase) condensation of vanillylamine, a phenyl propanoid intermediate with 8-methyl-nonenoic acid, a fatty acid derivative from leucine/valine pathway. Analysis of levels of 8-methyl-nonenoic acid and phenyl propanoid intermediates in high, medium, and low pungent *Capsicum* genotypes revealed that the 8-methyl-nonenoic acid pool plays a crucial role in determining the efficacy of capsaicin levels. Cerulenin-mediated inhibition of 8-methyl-nonenoic acid synthesis decreased the capsaicin biosynthesis in *Capsicum* cell suspension cultures. Similarly amino oxy acetate inhibited vanillylamine synthesis but failed to reduce capsaicin production. The mRNA transcript analysis of keto acyl synthase (KAS), a crucial enzyme involved in 8-methyl-nonenoic acid and an amino transferase (AMT), involved in vanillylamine biosynthesis was studied. The mRNA transcript analysis revealed the progressive developmental expression of KAS gene in the placenta during the ontogeny of the fruit, whereas AMT transcripts levels did not show significant differences. Hence, the study demonstrates the influence of 8-methyl-nonenoic acid and its possible regulatory role in capsaicin biosynthesis in *Capsicum* spp.

KEYWORDS: ***Capsicum*** **spp.; capsaicin synthase; vanillylamine; 8-methyl-nonenoic acid; mRNA transcripts; cerulenin; amino oxyacetate**

INTRODUCTION

Chilli (*Capsicum* spp.) is one of the major economical crops of *Solanaceae* grown for its pungency and carotenoid pigments (*1*). It is well documented that the pungency of *Capsicum* is due to capsaicinoids, the majority of which are capsaicin and dihydrocapsaicin. Capsaicin is mainly used as a spice, as food additives, and in pharmacological applications. As a medicine, capsaicin is known to kill some types of cancer cells (*2, 3*) and provide relief in arthritis and respiratory ailments (*4*). It is a counterirritant and an analgesic agent (*5*). Hot sauces and tomato-based salsa containing hot peppers have enjoyed strong gains in consumer acceptance in recent years and account for an estimated $500 million (U.S.) in annual sales (*6*). Exposure to capsaicinoids elicits a variety of physiological responses that include coughing and gagging, disorientation, erythema, skin reddening, lacrimation, temporary blindness, and intense burning sensation (*7*). It was also estimated that 15 million Americans carried these products for self-defense during 1996 (*8*).

There has been a lot of genetic diversity among *Capsicum* genotypes with respect to their pungency as a consequence of genotype and environment (*9, 10*). The inheritance of quantitative variation of capsaicinoid content was studied by means of biometrical analysis in different crosses (*11*). The capsaicin synthase (CS) assay has been demonstrated in placental tissues of *Capsicum* fruits (*12, 13*) (**Figure 1**). Although the first report on capsaicin biosynthesis mainly emphasized condensation of vanillylamine and fatty acid moieties by capsaicin synthase (*14*), to date there has been no report on the purification of capsaicin synthase and regulatory steps of the capsaicin biosynthetic pathway. However, there are reports on molecular identification of differentially expressed transcripts in high pungent and low pungent chillies (*15*), isolation of cDNA clones by suppression subtractive hybridization (*16*), and molecular mapping and QTL analysis for capsaicinoid content (*17*). Recently, *pun*1, an acyl transferase, was reported to be involved in capsaicin biosynthesis (*18*). The objective of this study was to unravel the importance of intermediates of capsaicin biosynthesis, which influence capsaicin production in various genotypes, and furthermore to understand the role of immediate precursors of capsaicin and enzymes involved in vanillylamine and 8-methyl-nonenoic acid synthesis.

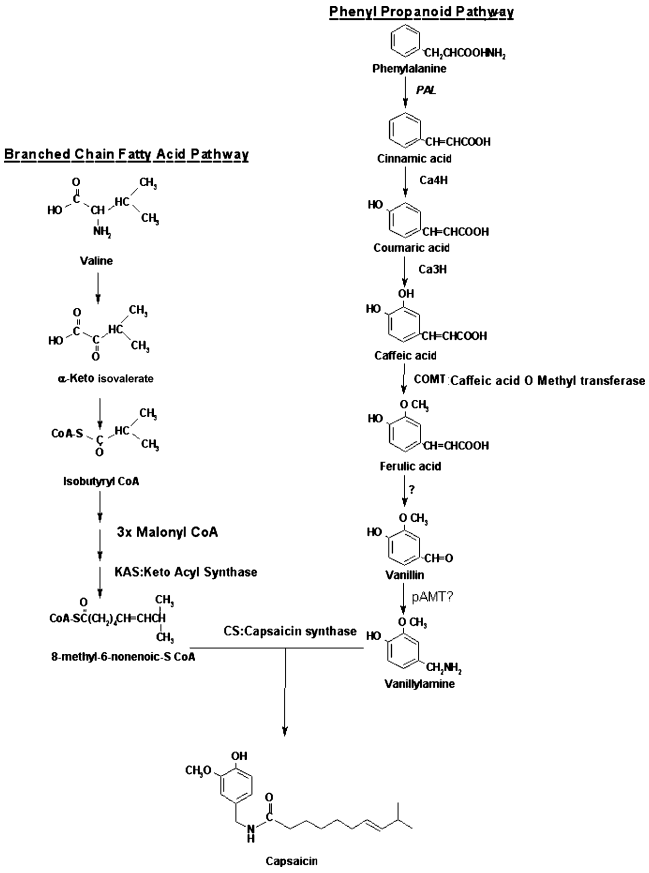

Figure 1. Capsaicin biosynthetic pathway. PAL, phenylalanine ammonia lyase; Ca4H, cinnamic acid 4 hydroxylase; Ca3H, coumaric acid 3 hydroxylase; CoMT, caffeic acid O methyl transferase; pAMT, putative amino transferase; CS, capsaicin synthase; KAS, keto acyl synthase.

MATERIALS AND METHODS

Chemicals. Capsaicin, dihydrocapsaicin, phenylalanine, cinnamic acid, coumaric acid, ferulic acid, cerulenin, DEPEC, BF_3, ascorbic acid, β-mercaptoethanol, ATP, Acetyl CoA, oligo-dT (18 mer) primer, ethidium bromide, Tris, and kinetin were purchased from Sigma Co., USA. 8-Methyl-nonenoic acid was purchased from Echemchina, China, whereas vanillylamine and amino oxyacetate were purchased from Aldrich Co., USA. NaOH, NaCl, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, magnesium chloride, 2,4-D, sucrose, and agarose were purchased from Himedia, Mumbai, India. RNA extraction, RNase Zap, M-MuLV reverse transcriptase, and DNase were purchased from Ambion, USA. *Taq* DNA polymerase was purchased from MBI Fermentas, Lithuania. All of the solvents used for HPLC and GC were purchased from Merck, Germany.

Germplasm Collection. Viable seed samples of *Capsicum* with wide genetic background were collected from different sources. The *Capsicum annuum* var Arka Abhir were received from Indian Institute of Horticultural Research, Bangalore, India. *Capsicum frutescens* land races, AW-2 (Bird eye Chilli), was collected from Biligiri Rangana hills, Karnataka state, and M-4 was from Manipur state, India.

Callus and Cell Suspension Cultures. In-vitro seedlings of *Capsicum frutescens* were raised in half strength Murashige and Skoog (MS) medium (*19*). Callus cultures were raised in full strength MS solid medium supplemented with 2,4 D (2 mg L^{-1}) and kinetin (0.5 mg L^{-1}) and 3% sucrose. The cultures were regularly subcultured and transferred to a 150 mL conical flask containing 40 mL of MS liquid medium supplemented with 2,4 D (2 mg L^{-1}) and kinetin (0.5 mg L^{-1}) and 3% sucrose. Cultures were grown on a rotary shaker at 100 rpm with a 12-h photoperiod at 25 ± 2 °C. The media pH was adjusted to 5.7 before autoclaving. Cells were harvested by filtration. Control and treated cell suspension cultures of *Capsicum frutescens* were harvested and dried at 60 °C until it attained constant weight. Fresh and dry weight of cells were determined and recorded. The capsaicin was extracted from callus as well as from the medium (*20*).

Quantification of Phenyl Propanoid Intermediates and Capsaicinoids. *Capsicum* fruits/callus of different genotypes were harvested after anthesis and dried at 60 °C until it attained constant weight. The dried fruits were homogenized in a mortar containing quartz sand with acetonitrile (1:10 w/v). The extract was centrifuged at 10 000*g* at 4 °C for 15 min, and the pellet was discarded. The aliquots were evaporated to dryness in vacuo, and extracts were resuspended with 1.0 mL of HPLC grade methanol. The samples were centrifuged at 6000*g* for 15 min before injecting to HPLC (Shimadzu, CR-7A). Capsaicinoids and intermediates of phenyl propanoid pathway were quantified by HPLC (*21*). The mobile phase of the linear gradient of 0−100% acetonitrile in water with pH 3.0 for 35 min and 100% was maintained for an additional 2 min. The detection was at 236 nm, and the flow rate was maintained at 1 mL/min. A C-18 column of 250 × 4.6 mm and 5 μm diameter was used. The reagents used were of HPLC grade. 5 and 10 μL of samples and standards were injected thrice, and the mean area was calculated.

Quantification of 8-Methyl-nonenoic Acid. One gram of dried fruits/suspension cells were homogenized in a mortar containing quartz sand with petroleum ether (1:20 w/v). The extracts were concentrated in vacuo at 40 °C to dryness. Oils were saponified with NaOH (0.5 N), and free acids were converted to methyl esters with boron trifluoride (BF_3) and heated for 5 min at 65−70 °C. Saturated NaCl (0.5 mL) was added followed by 3 mL of hexane. The hexane fraction was used for GC and GC−MS (Perkin-Elmer). The DB-1 column (Cat # 122-1032, J & W Scientific Co. USA) was used in the study. The length of the column was 30 m, whose internal diameter and film depth was 0.25 mm and 0.25 μ, respectively. The initial temperature of the capillary column (DB-1) was maintained at 80 °C the first 5 min and increased up to 200 °C at the 20th min with an increment of 5 °C per min. The helium flow rate was 1 mL min^{-1}. Spiking was carried out for further corroboration.

Incorporation of Cerulenin (2,3-Epoxy-4-oxo-7,10-dodecadienamide) into Suspension Cell Cultures. Filter sterilized cerulenin (*31*) was incorporated into 10 days old suspension cultures at 2.5, 5, and 10 μM. The capsaicin, vanillylamine, and 8-methyl-nonenoic acid were estimated in cell suspension cultures 20 days after cerulenin treatment.

Incorporation of Amino-oxy Acetate to Suspension Cell Cultures. Similarly, filter sterilized amino-oxy acetate (*22*) was incorporated into 10 days old suspension cultures at 0.01, 0.05, and 0.1 mM concentrations. The capsaicin, vanillylamine, and 8-methyl-nonenoic acid were estimated in cell suspension cultures 20 days after the incorporation of amino oxyacetate.

Incorporation of Precursors to Suspension Cell Cultures. Ferulic acid, vanillylamine, nonenoic acid, and a combination of vanillylamine and nonenoic acid were incorporated into 10 days old suspension cultures of Arka Abhir at 1, 2.5, and 5 μM concentrations. The capsaicin was extracted and estimated as mentioned above on the 20th day after precursor treatment.

Enzyme Assay. The activity of capsaicin synthase was assayed (*23*) with modification using HPLC to facilitate nanomoles detection of capsaicin. The cell free extracts of *C. frutescens* were extracted from 10 g of placenta from freshly harvested fruit with 0.1 M potassium phosphate buffer, pH 6.8, with 100 mg of ascorbic acid and 5 mM β-mercaptoethanol. The homogenate was centrifuged at 8000*g* for 30 min at 4 °C. The supernatant was subjected to different ammonium sulfate fractionation (40−60%) and dialyzed. The dialyzed extract was used as the enzyme source. The ammonium sulfate fractions were dialyzed against 0.1 M potassium phosphate buffer and made free of ATP and other nonproteinaceous components. Protein was estimated by Bradford's method (*24*).

The CS reaction mixture contained 0.5 M potassium phosphate buffer pH 6.8, 1 μM $MgCl_2$ and ATP, 5 μM vanillylamine, 8-methyl-nonenoic acid, acetyl CoA, and 100 μg of protein. The reaction mixture was incubated for 2 h at 37 °C and terminated by 0.1 mL of methanol. The reaction mixture was taken into the chloroform fraction. The chloroform fraction was evaporated in vacuo and resuspended with 0.1 mL of methanol, and this fraction was injected into HPLC. An in-vitro assay was also devised to study the relative contribution of enzyme, ATP,

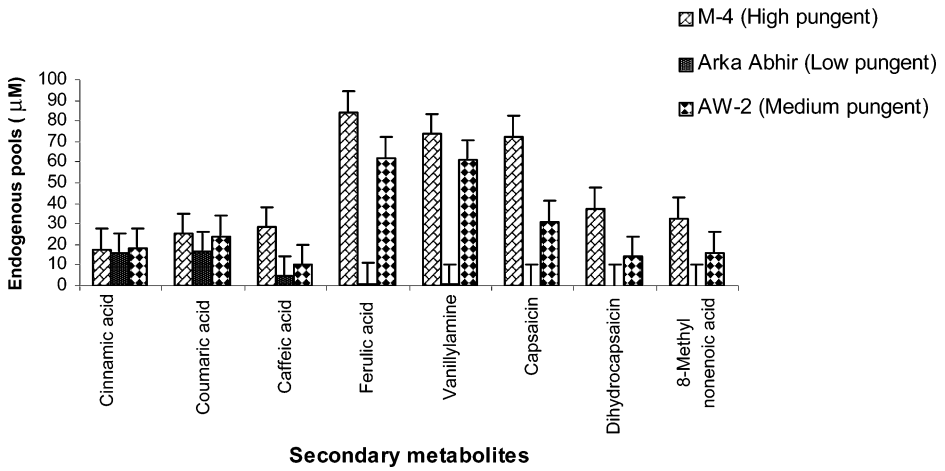

Figure 2. Levels of phenyl propanoid intermediates, capsaicinoids, and 8-methyl-nonenoic acid in low, medium, and high pungent *Capsicum* genotypes after 35 days of anthesis.

$MgCl_2$, and CoA on capsaicin biosynthesis by eliminating one of the components of the reaction mixture. The treatment, which received all of the components, served as a positive control, and the entire component with heat denatured enzyme extract was treated as a negative control.

Expression Analysis of AMT and KAS by RT-PCR. In the capsaicin biosynthetic pathway, placental specific amino transferase (AMT) (*15*) and keto acyl synthase (KAS) (*25*) are known to be involved in vanillylamine and 8-methyl-nonenoic acid production, respectively. To study the mRNA transcript of these two genes, total RNA was extracted using the total RNA extraction kit. To avoid possible RNase contamination, all plasticware was treated with 0.1% DEPEC, and the working area, electrophoresis tank, and other required materials were treated with RNase Zap. *Capsicum* fruits from high pungency genotype were harvested at different stages of fruit development and frozen in liquid nitrogen followed by immediate RNA extraction. The quality and concentration of RNA were checked on denaturing agarose gel and by absorbance measurements at 230, 260, and 280 nm in a UV spectrophotometer. All of the RNA samples were subjected to DNase treatment to avoid possible artifact amplifications from contaminant genomic DNA.

The KAS and AMT gene specific primers were designed using Primer3 software (*26*). A control PCR was run on extracted RNA samples to check for the absence of genomic DNA. First-strand cDNAs were synthesized from 2 μg of total RNA in 20 μL final volume, using M-MuLV reverse transcriptase and oligo-dT (18 mer) primer following manufacturer's instructions. The RT-PCR reaction was stopped in the early exponential phase (20 cycles) to maintain initial differences in target transcript quantities (exponential phase of amplification). PCR reactions were subjected to 20 cycles at 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s with *Taq* DNA polymerase (MBI Fermentas, Lithuania). The primers for KAS were forward-5′GCTTGTGCAACTGCCAATAT3′ and reverse- 5′ GGATGAAGCCATCCTGTTGT3′ and for AMT forward 5′GCAACATTAGGGGGAAGTGA 3′ and reverse 5′ CTGCTCCTAGGACTGGTTCG 3′. An aliquot of 10 μL from each PCR reaction was fractionated on a 1.5% (w/v) agarose gel in Tris-acetate EDTA buffer. Ethidium bromide (0.5 μg/L) stained gels were photographed with a Digital Imaging System (HeroLab, Germany). The transcriptional abundance of the amplicons of KAS and AMT was estimated by intensity histogram. α-Tubulin, used as an internal constitutive control, was amplified using primers forward 5′-CTGTCAACGACCCCTTCATC-3′ and reverse 5′-CCTGTTGTCGCCAACGAAGTC-3′.

Statistical Analysis. The mean and standard deviation were calculated according to Tukey's method (*27*).

RESULTS

Levels of Phenyl Propanoid Intermediates, Capsaicinoids, and 8-Methyl-nonenoic Acid. The estimation of capsaicin intermediates revealed a genotype-specific difference in the capsaicin biosynthesis pathway profile. There was not much difference among different categories of *Capsicum* varieties especially for cinnamic acid and coumaric acid, whereas the levels of caffeic acid varied significantly among the different categories of *Capsicum* genotypes. The amount of caffeic acid was almost 3-folds more in the high pungent genotype M-4 (28 μM) over medium pungent genotype AW-2 (10 μM) and almost 6-folds more than low pungent variety Arka Abhir (4.5 μM).

The level of ferulic acid in high pungent genotypes M-4 (84 μM) was 84 times more than that of the low pungent variety Arka Abhir (0.8 μM) and 1.35 times higher than that in medium pungent genotype AW-2 (62 μM). A similar trend continued with the levels of vanillylamine where in the high pungent genotype M-4 (73 μM) had 147 times more vanillylamine than low pungent genotype Arka Abhir (0.5 μM) and 1.2-fold more than medium pungent genotype AW-2 (61 μM) (**Figure 2**).

The high pungent genotype M-4 had the highest 8-methyl-nonenoic acid content (32 μM), which is 652 times more than that of the low pungent genotype Arka Abhir (0.05 μM) and 1.16-fold more than that of the medium pungent genotype AW-2 (28 μM). Correspondingly, the capsaicinoid content was highest in M-4 (72 and 37 μM of capsaicin and dihydrocapsaicin, respectively), whereas the lowest was detected in Arka Abhir (0.1 μM each of capsaicin and dihydrocapsaicin) (**Figure 2**). Thus, the correlation of capsaicin levels with 8-methyl-nonenoic acid was very evident in the genotypes, demonstrating a strong correlation between the capsaicin and 8-methyl-nonenoic acid pool among different genotypes. So far, there are no reports on the role of endogenous pool of fatty acid 8-methyl-nonenoic acid, one of the substrates of capsaicin biosynthesis. This is the first report on the quantification of 8-methyl-nonenoic acid in *Capsicum* fruits and revealing its probable role in capsaicin biosynthesis.

Effect of Cerulenin (Fatty Acid Synthase Inhibitors) on Capsaicin Biosynthesis. Cerulenin, a known inhibitor of fatty acid pathway, was administered to cell cultures to study its influence on 8-methyl-nonenoic acid and in turn on capsaicin production. Significant reduction in 8-methyl-nonenoic acid (up to 80%) and capsaicin levels (up to 86%) was noticed when cerulenin was added at 5 μM concentration (**Figure 3**).

Effect of Amino Oxy Acetate (Amino Transferase Inhibitor) on Capsaicin Biosynthesis. Amino oxy acetate was found efficient in the inhibition of vanillylamine. Maximum reduction in vanillylamine (up to 80%) was noticed when amino oxy

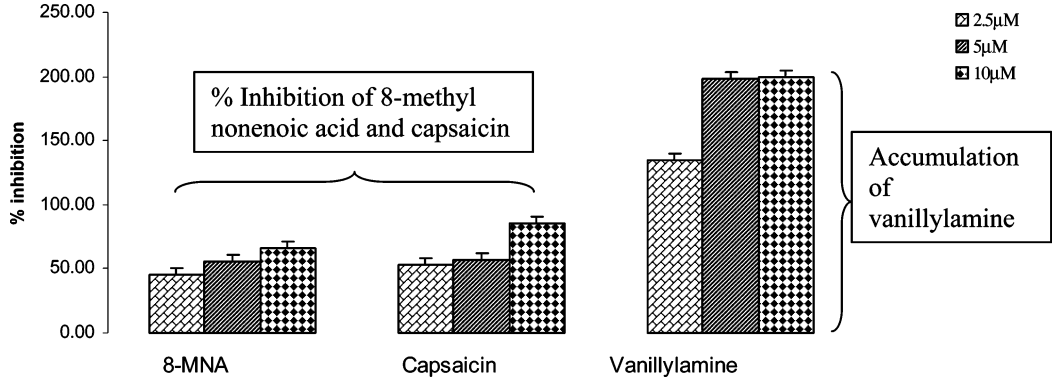

Figure 3. Effect of cerulenin on capsaicin biosynthesis.

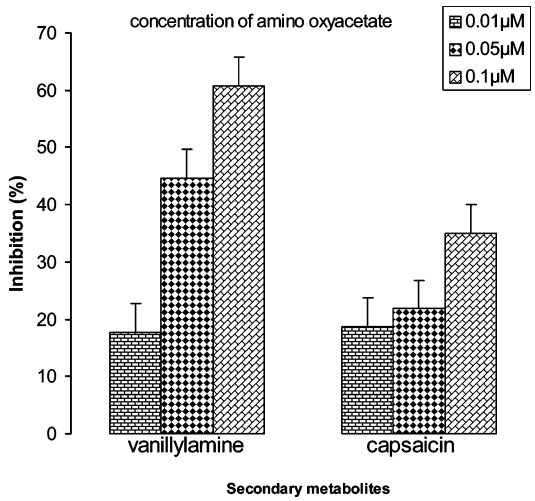

Figure 4. Effect of amino oxyacetate on capsaicin biosynthesis.

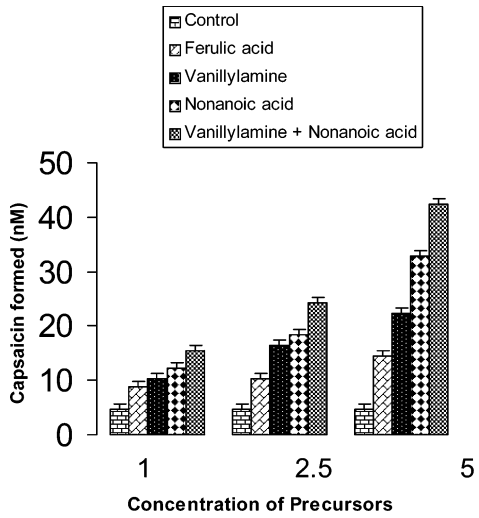

Figure 5. Effect of precursors on capsaicin biosynthesis in cell suspension cultures. Ferulic acid, vanillylamine, 8-methyl-nonenoic acid, and combination of vanillylamine and nonenoic acid were incorporated to 10 days old suspension cultures of Arka Abhir at 1, 2.5, and 5 μM concentrations. Maximum capsaicin was produced when vanillylamine and 8-methyl-nonenoic were incorporated exogenously.

acetate was added at 0.1 μM concentration (**Figure 4**). Interestingly, the capsaicin was still produced at 35% even with 80% reduction of levels of vanillylamine. This clearly indicated that the vanillylamine pool is not the limiting factor in capsaicin biosynthesis.

Effect of Precursors on Capsaicin Biosynthesis. Maximum capsaicin (42 nM) was produced when vanillylamine and nonenoic were supplied together. When the precursors were supplied individually, maximum capsaicin (32 nM) was produced with the supply of 8-methyl-nonenoic acid, whereas minimum capsaicin (14 nM) was produced when ferulic acid was supplied (**Figure 5**).

Enzyme Assay. There was linearity in the assay procedure for capsaicin synthase. The enzymatic formation of capsaicin was measured at varying concentration of placental protein extracts. The specific activity of capsaicin synthase was constant at 100 mg of protein extracts. The coefficient of variation (standard deviation/mean *100) of the enzyme assay was 7%. One unit of capsaicin enzymatically formed was defined as 1 nanomol of capsaicin produced by milligram of protein per hour. The specific activity of capsaicin synthase was 18.17 units/mg protein/h. The absence of either 8-methyl-nonenoic acid or vanillylamine in the reaction mixture did not produce capsaicin. The results revealed that ATP, $MgCl_2$, and CoA are absolutely required for CS activity.

Expression Analysis by RT-PCR. The gene specific primers of KAS (AF085148) and AMT (AF085149) were used to study genotype and developmental expression. The increase in expression of KAS correlated with levels of pungency in the different genotypes studied. During the ontogeny of the fruit, KAS levels were also increased. The maximum transcripts of KAS were observed in 35-day-old fruit. In contrast, the mRNA transcripts of AMT were almost the same during the ontogeny of the fruit.

DISCUSSION

There has been a lot of ambiguity in capsaicin biosynthesis with regard to putative capsaicin synthase, which brings about condensation between 8-methyl-nonenoic acid and vanillylamine to produce capsaicin. The results clearly demonstrated that between the two substrates for capsaicin biosynthesis, vanillylamine is found in abundance but 8-methyl-nonenoic acid is the limiting factor, which determines the efficacy of capsaicin biosynthesis. This study mainly envisaged the regulatory steps of capsaicin biosynthesis. It is evident from the endogenous pool data that levels of phenylalanine, cinnamic acid, coumaric acid, and caffeic acid do not differentiate the genotypes for pungency.

Yet in general the level of ferulic acid was more in the genotypes having more vanillylamine (**Figure 2**). However, it could be interesting to note that a switch in the flux of intermediates from ferulic acid is in the favor of the synthesis of capsaicinoids, although ferulic acid is also involved in the synthesis of cinnamoyl glycosides, lignin biosynthetic pathway, and phenolic glycosides (*1*). This may be due to the fact that ferulic acid is one of the important precursors in capsaicin biosynthesis that acts as a substrate for vanillin biosynthesis, which proceeds to vanillylamine by the action of amino

transferase (*16*). Involvement of ferulic acid in lignin biosynthesis through ramification of sinapic acid biosynthesis is also preordained (*28*).

The levels of vanillylamine and 8-methyl-nonenoic acid, the immediate precursors in capsaicin biosynthesis, were highest in M-4, whereas Arka Abhir had the lowest amounts of vanillylamine and 8-methyl-nonenoic acid. The increase in capsaicin levels in genotypes well correlated with the levels of vanillylamine and 8-methyl-nonenoic acid. It could also be noticed that between M-4 (high pungent) and AW-2 (medium pungent), the levels of vanillylamine were almost equimolar but the levels of 8-methyl-6-nonenoic acids were almost 2-folds more in M-4 (**Figure 2**).

From RT-PCR studies, it was evident that KAS expression correlated with the level of capsaicin production. However, there was no significant difference in AMT expression during the ontogeny of fruit. These studies confirmed the role of endogenous levels of vanillylamine and 8-methyl-nonenoic acid and the role of the KAS and AMT regulated expression pattern during the ontogeny of *Capsicum* fruit.

In the precursor feeding experiment, it was evident that significantly high capsaicin was produced when 8-methyl-nonenoic acid was supplied individually and in combination with vanillylamine. This suggests that 8-methyl-nonenoic acid is very close in pathway, leading to capsaicin biosynthesis, and although vanillylamine is present in abundance, the quantity of 8-methyl-nonenoic acid determines the pungency in placental tissues of *Capsicum*. In our earlier studies (*20*, *21*) and also from studies of Kang et al. (*29*), capsaicin is found to be leached out into media. In our study, the culture media was also analyzed for capsaicin. The data presented here are the sum of capsaicin in cells as well as in media.

In a separate study, wherein each cofactor and their combination were excluded from the CS assay mixture, ATP and $MgCl_2$, there was a drastic reduction in the levels of capsaicin production, suggesting the enzymatic capsaicin production is cofactor dependent. Similar results were observed from the studies of Fujiwake et al. (*12*). In their study, they predicted the involvement of two enzymes in the formation of capsaicin. Yet the functionality of these two enzymes in capsaicin formation is still unclear. However, *pun*1, an acyl transferase, is reported to be crucial in capsaicin biosynthesis as virus induced gene silencing of *pun*1 resulted in lesser accumulation of capsaicinoids (*18*). From these studies, it is very much clear that there is an involvement of an acyl transferase in capsaicin biosynthesis, which is also in agreement with the studies of Kim et al. (*16*).

Further in inhibitor studies, fatty acid synthase inhibitor cerulenin reduced 8-methyl-nonenoic acid because it is biosynthesized through fatty acid synthase-II (FAS), acyl carrier protein (ACP), and keto acyl synthases (KAS) (*25*). Moreover, cerulenin is reported to be a potent inhibitor of keto acyl synthase in *Spinacia oleracea* (*30*, *31*), which may be responsible for reduction in levels of 8-methyl-nonenoic acid and ultimately capsaicin. Interestingly, the levels of vanillylamine accumulated in cerulenin treated cultures, indicating the nonavailability of 8-methyl-nonenoic acid for enzymatic condensation to produce capsaicin by capsaicin synthase. Amino oxy acetate, aminotransferase inhibitor, inhibited the production of vanillylamine up to 80%. Vanillylamine is known to be produced from vanillin by amino transferase in a pyro phosphate-dependent manner (*15*). Even with 80% inhibited levels of vanillylamine, capsaicin was produced to the extent of 35%, whereas cerulenin inhibited 8-methyl-nonenoic acid (66%) and drastically reduced capsaicin production (up to 85%). These results shows that among the two pathways involved in capsaicin biosynthesis, the fatty acid pathway is more crucial, which determines the pungency levels of the genotypes.

From enzymatic studies, the quantification of endogenous pool of phenyl propanoid intermediates, 8-methyl-nonenoic acid, the impact of cerulenin and amino oxyacetate on capsaicin biosynthesis, and also from the mRNA expression profile studies, we have evidence that 8-methyl-nonenoic acid plays a crucial role in determining the pungency of *Capsicum* genotypes.

ACKNOWLEDGMENT

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