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Effect of invertase activity from strain of *Cladosporium cladosporioides* on physiological process of cut flowers

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ABSTRACT

The filamentous fungus *Cladosporium cladosporioides* was a good producer of invertase under submerged fermentation (SmF). The production of invertase enzyme under SmF was enhanced on fourth day of incubation at an optimum pH 4.0 and temperature 30^0C respectively. The enzyme was purified by DEAE Cellulose chromatography with 6.35 folds and showed a molecular weight of 61kDa by SDS-PAGE. Treatment of cut flowers with cytokinins is found to be beneficial in delaying senescence processes but the response to cytokinin application varies depending on cultivar, stage of flower development and type of cytokinin. Treatment of cut roses with Glean-75 + sucrose and invertase were associated with the phases of development resulted in a longer vase life, successful opening and good quality of the flowers. This finding demonstrates that extracellular invertase is required for the delay of senescence by cytokinins.

Keywords: *Cladosporium cladosporioides,* molecular weight, invertase, purification, DEAE-Cellulose, cut-flowers

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INTRODUCTION

Cytokinin is a group of plant hormones that promote cell division and play a major role in the regulation of various biological processes associated with active growth, metabolism and plant development. Because these processes are associated with an enhanced demand for carbohydrates, a link to the regulation of assimilate portioning [1], sink strength [2], and source-sink relations [3] has been suggested. The photo assimilates produced in the source organs are transported into the sink organs mostly in the form of sucrose. An apoplasmic phloem unloading of sucrose is mandatory in simplistically isolated tissues, such as embryos or stomata, and seems to be characteristic for actively growing tissues [4] and, thus, under conditions that may be under the control of cytokinins.

In apoplastic unloading pathways, sucrose is released from the sieve elements of the phloem into the apoplast by a sucrose transporter, where it is irreversibly hydrolyzed by an extracellular invertase ionically bound to the cell wall [5]. The extracellular invertase has a crucial function both in source-sink regulation and for supplying carbohydrates to sink tissues, being considered as a central modulator of sink activity [6]. It has been shown that extracellular invertases are up regulated by several stimuli that affect carbohydrate requirements, including growth stimulating phytohormones [7].

The extracellular invertase activity is usually high in tissues with an elevated cytokinin concentration. Stimulation of invertase activity may be considered as way for ensuring supply of energy for bud growth, delay of ageing, enhancement of defense mechanism giving an increase in energy supply under stress conditions, thus contributing to an extension in vase-life. Senescence is a type of programmed cell death that constitutes the final stage of plant development in which the nutrients get remobilized to other parts of the plant. Senescence is accompanied by a decline in cytokinin content. Exogenous application of cytokinins or an increase of the endogenous concentration delays senescence and causes nutrient mobilization. It has been proved that the enzyme invertase activates the production of cytokinin thereby delaying the process of senescence [8-10].

The present study was started as a survey of new species to learn their capacity to produce invertase when grown in shaken cultures. The study was conducted to explore the effect of invertase on cut rose flowers.

MATERIALS AND METHODS

Organism and inoculum preparation

Fungal strains were isolated from soil of sugarcane field Coimbatore, India by dilution plate method. Culture was screened for invertase enzyme production and fungal strain Cladosporium cladosporioides selected for the production of invertase was prepared from 4 days old slant culture.

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Fermentation condition

The medium used for enzyme production under submerged fermentation comprised of (gm/L): sucrose 20, yeast extract 10, ammonium sulphate 1.0, magnesium sulphate 0.75, potassium dihydrogen phosphate 3.5, pH 5.0. Cultivation was carried out in 250 ml Erlenmeyer flasks each containing 50 ml of sterile medium. After inoculation (10^6 spores/ml), the flasks were incubated at 30 °C for seven days in an incubator shaker at 125rpm. At the end of fermentation, the supernatant was harvested by centrifugation at 10,000 rpm for 10 min (4 ^{o}C) and was used as crude enzyme extract.

Enzyme assay

Invertase activity was determined using the method of Miller [11]. One unit of invertase (IU) is defined as the amount of enzyme which liberates 1µ moles of glucose/minute/ml under the assay condition

Purification of invertase

Crude extract was precipitated by 70% saturation with ammonium sulphate and then dialyzed against 100mM Tris phosphate buffer (pH 7.5) for 24 hours at 40 C. the filtrate was loaded onto a DEAE-cellulose chromatographic column (25 cm * 2.6 cm) equilibrated with Tris-HCl buffer, 100mM, pH 7.5.The enzyme was eluted with a linear salt concentration gradient (NaCl, 0-0.4 M) in the same buffer and 3.0 ml fractions were collected at a flow rate of 20 ml per hour. SDS-PAGE electrophoresis was carried out and molecular weight was determined. The protein content was estimated by the method of Lowry et al., [12]. The purified enzyme is used for further studies.

Effect of invertase activity on physiological process of cut flowers

The senescence in cut flower (rose) and impact of invertase enzyme on the physiological process was assessed.

The experiment involved the following different treatments.

T_1 - Rose petals + Water
T_2 - Rose petals + Water + Invertase
T_3 - Rose petals + Sucrose + Invertase
T_4 - Rose petals + Chlorsulfuron + Invertase
T_5 - Rose petals + Sucrose + Chlorsulfuron + Invertase

The rose petals were kept in a beaker with the above treatments for 10 days. The first signs of wilting, vase life and colour change in petals were observed on these days. The

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invertase activity and protein were also observed in these petals following the method of Sumner and Howel, [13] and Lowry et al., [12] respectively.

RESULTS AND DISCUSSION

Invertase production by C. cladosporioides was studied in shaken flask culture technique by inoculating 10^6 spores/ml of fermentation medium (CD medium). The CD medium was inoculated with the fungal strain and incubated for various time intervals (1-7 days). The highest production was observed on fourth day (23.2 IU/ml) of incubation using C. cladosporioides (Figure.1)

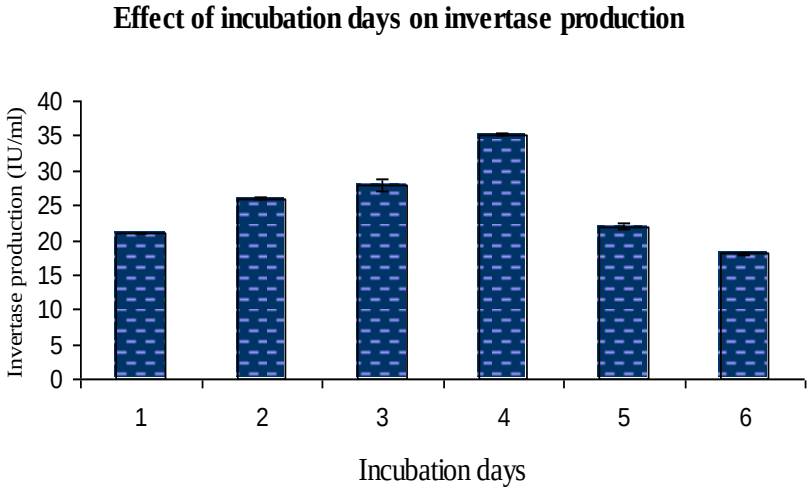

Figure 1: Results are mean of three independent determinations. Bars correspond to standard deviation.

Guimaraes et al., [14] reported that for *Aspergillus ochraceus*, β-D-fructofuranosidase reached its highest level when supplemented with sugarcane bagasse at 96 hours i.e on 4th day at 40º C under orbital agitation of 100 rpm. The maximum invertase production by Aspergillus oryzae on 4th day incubation at pH 5.5 was reported by Shankar and Mulimani, [15]. Similar result was shown by Yun et al., [16] for *Aspergillus pullulans*.

The crude enzyme of C. cladosporioides was precipitated at 70% saturation of ammonium sulphate with 1.52 fold purification and 63.9% recovery with a specific activity 47.25 U/mg. After dialysis, passage from DEAE cellulose column further purifies the enzyme to 6.35 fold with 3.96 % recovery (Table 1). Concurrently, the eluted sample was subjected to SDS PAGE and the molecular weight was determined which was found to be 61kDa (Fig. 2). The homogenate of the dialysate was checked by SDS-PAGE was performed as discussed by Laemmli, [17]. Our result was in consonance with the work of Guimaraes et al., [14] who purified the enzyme to 7.1 fold with a recovery of 24%, by two chromatographic steps in DEAE-cellulose and sephacryl s-200, in Aspergillus ochraceus.

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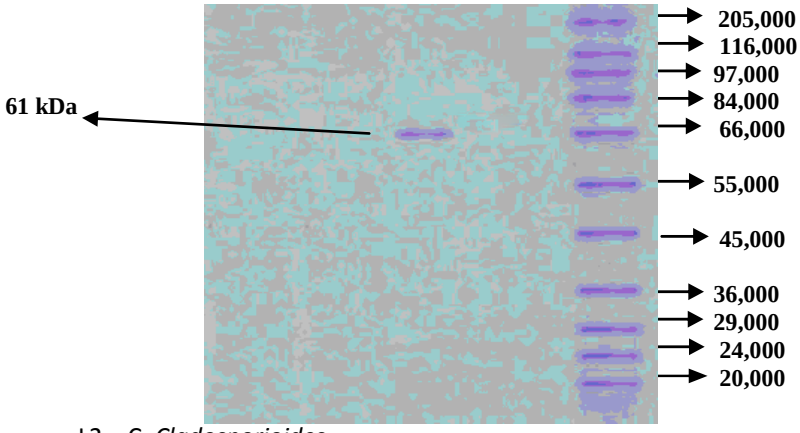
Shaheen et al., [18] reported that 20.25-fold purification and 5.53% recovery in Fusarium species. Ishimoto et al., [19] purified invertase from Clostridium perfringens AS40-75 by DEAE-cellulose chromatography, gel filtration through sephadex G-150 and hydroxylapatite chromatography and the maximum recovery was with ammonium sulphate i.e. 91% with specific activity of 1.42.

Table: 1. Purification and recovery of invertase from *C. cladosporioides*

Steps	Invertase activity (U)	Total protein (mg)	Specific activity (U /mg)	Purification (fold)	Recovery (%)
Crude extract	9940	320.64	31	1	100
70% Ammonium sulphate precipitation	6360	134.60	47.25	1.52	63.9
Dialysis	469.20	9.08	51.75	1.67	4.72
DEAE Cellulose Column Chromatography	393.75	1.99	197.50	6.35	3.96

Molecular weight SDS-PAGE



The molecular weight of invertase for many organisms was reported by many workers. In our present study, the molecular weight was determined by SDS-PAGE and was found to be 67 C. cladosporioides. Ettalibi and Barathi, [20] reported the molecular weight of invertase in A. ficcum to be 84kDa. Hang et al., [21] reported that the molecular weight of invertase in the yeast (Saccharomyces) was 57kDa.

The impacts of invertase enzyme on the physiological process of cut flower were studied by treating the rose petals with partially purified invertase enzyme, sucrose and chlorsulfuron solution. The results are shown in Table 2.

The experimental findings revealed that T5 exhibited a significant delay in wilting of petals (6.62 ± 1.25), increased vase-life (7.3 ± 1.14) and colour change was also observed to be

delayed in this treatment (Table 2). The result obtained for the invertase activity and protein are depicted in (Table: 3) were also higher in T5 (39.87 IU/ml and 5.89 mg/ml).

Table 2. Effect of sucrose, chlorsulfuron and invertase on post-harvest life of rose petals

Treatment	Solutions	First signs of wilting (day)	Vase-life (day)	Petals (day) Colour change
T1	Rose petals+water (control)	2.84 ± 0.32^{a}	4.0 ± 0.31^{a}	1 – 2
T2	Rose petals+water+ invertase	2.95 ± 0.44^{a}	4.1 ± 0.43^{a}	2 – 3
T3	Rose petals+ sucrose+invertase	4.42 ± 0.59^{b}	5.2 ± 1.19^{b}	3
T4	Rose petals+ chlorsulfuron+invertase	3.45 ± 0.66^{c}	4.2 ± 0.72^{a}	3-4
T5	Rose petals+sucrose chlorsulfuron+invertase	6.62 ± 1.25^{d}	7.3 ± 1.14^{c}	6

Values are Mean ± SD of three samples. Means followed by a common superscript letter are not significantly at 5% level by using DMRT analysis

Table 3. Enzyme activity and protein concentration of rose petal extract

Treatment	Solutions	Invertase activity(iu/ml)	Protein concentration(mg/ml)
T1	Rose petals+water (control)	9.83 ± 1.08^{a}	1.14 ± 0.26^{a}
T2	Rose petals+water+ invertase	19.78 ± 1.57^{b}	2.55 ± 0.79^{b}
T3	Rose petals+ sucrose+invertase	23.56 ± 1.93^{c}	3.72 ± 0.83^{c}
T4	Rose petals+ chlorsulfuron+invertase	25.76 ± 2.05^{d}	4.22 ± 1.02^{d}
T5	Rose petals+sucrose chlorsulfuron+invertase	39.87 ± 2.68^{e}	5.89 ± 1.48^{e}

Values are Mean ± SD of three samples. Means followed by a common superscript letter are not significantly at 5% level by using DMRT analysis

The results indicate that treatment with solutions containing sucrose and invertase or sucrose + chlorsulfuron + invertase caused an enhancement of enzyme activity. The protein activity has also observed to be increased in the treatments with sucrose, chlorsulfuron and invertase (Fig. 3).

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A. Pulse Treatment of Petals

B. Incubated in various blossoming solutions

A B C D E

A - Solution with rose petals + water

B - Solution with rose petals + water + invertase

C - Solution with rose petals + chlorsulfuron + invertase

D - Solution with rose petals + sucrose + invertase

E - Solution with rose petals + sucrose + chlorsulfuron + invertase

C. Petals after 10 days incubation

The activity of invertase in present study is in accordance with Yakimova et al., 1996 [8] where it was observed that treatment with sucrose and chlorsulfuron exhibited an enhanced activity.

Cut rose flowers are susceptible to water stress because the water balance of petals is easily disturbed after harvesting. Rose flower are also often exposed to biotic stress which causes vascular occlusion of the flower stem beneath flower head [22]. The observed increase in invertase activity by chlorsulfuron and sucrose could be part of an enhancement of defense mechanisms giving an increase in energy supply under stress conditions, thus contributing to the extension in Vase-Life [8].

Mayak and Dilley, [23] have observed that petals of roses treated with sucrose and ethephon contained more protein than that of control. A delayed wilting of rose petals was observed (6th day) when they were kept in solutions containing sucrose, chlorsulfuron and invertase. It was followed by the solution consisting sucrose and rose petals.

The Vase-Life of petals was extended when kept in sucrose, chlorsulfuron and invertase compared to control. The colour change in petals was delayed by the treatment with sucrose, chlorsulfuron and invertase (4th day) compared to control. The treatment with chlorsulfuron and invertase also exhibited similar results (3 - 4 days).

The result of Yakimova et al., [8] also depicts that treatment with sucrose and chlorsulfuron had significant retention of leaf yellowing, extension of rose flowers longevity over control. The treatment also improved the capability of rose flowers.

The addition of sugars in Vase solutions is essential for a good flower development and facilitates the movement and utilization of substances with a nature of cytokinins [10, 24]. This might be a reason for the higher effect of combination of glean-75 and sucrose [8].

CONCLUSION

This may provide a clue to a better understanding of the role of invertase in the response of cut flowers to stress factors. In our attempt to elucidate the relationship between cytokinin and primary metabolism, the observed increase of invertase activity in the delayed-senescence in the rose petals supported a link among cytokinins, invertases and carbohydrate partitioning for the delay of senescence. The findings demonstrate that an increase in invertase activity is not only sufficient to cause a delay of senescence, but that this key enzyme of an apoplasmic phloem unloading pathway is an essential component of the molecular mechanism of delay of senescence by cytokinins.

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REFERENCES

[1] Brenner ML, Cheikh N. In Plant Hormones, P.J. Davies, ed (Dordrecht, The Netherlands: Kluwer Academic Press) 1995; 649–670
[2] Kuiper D. Plant Cell Environ 1993; 16: 1025–1026
[3] Roitsch T, Ehness R. Plant Growth Regul 2000; 32: 359–367
[4] Eschrich W. Ber Dtsch Bot Ges 1980; 93: 363–378
[5] Roitsch T, Tanner W. Bot Acta 1996; 109: 90–93
[6] Tang GQ, Lu scher M, Sturm A. Plant Cell 1999; 11: 1–14

[7] Roitsch T, Balibrea ME, Hofmann M, Proels R, Sinha AK. J Exp Bot 2003; 54: 513–524
[8] Yakimova E, Kapchina- Toteva V, Alexieva V, Sergiev I, Karanov E. Bulg J Plant Physiol 1996; 22: 74-87
[9] Balibrea Lara ME, Garcia Rainer Ehneb MG, Lee TK, Proels R, Roitsch WT. The plant cell 2004; 16: 1276-1287
[10] Paulin A. Acta Hort 1986; 181: 183-193
[11] Miller GL. Anal Chem 1959; 31: 426-428
[12] Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. J Biochem 1951; 193**:** 265-275
[13] Sumner JB, Howell SF. J BioChem 1935; 108: 51-54
[14] Guimaraes LHS, Terenzi HF, Lourdes MD, Jorge JA. J Enzyme and Microbial Technology 2007; 42: 52-57
[15] Vitolo M, Durante MA, Pellegrim MB. J Ind Microbiol 1995; 15: 75-79
[16] Shankar SK, Mulimani VH. Bioresource technology 2007; 98: 958-961
[17] Laemmli UK. Nature (London) 1970; 277: 680-685
[18] Shaheen I, Haq TA, Bhatti N. J Food Science and Tech 2007; 43: 1152-1158
[19] Ishimoto M, Nakamura A. J Biosci Biotech Biochem 1997; 64: 599-603
[20] Ettalibi M, Baratti JC. Appl microbial biotech 1987; 26: 13-20
[21] Hang YD, Splittstoesser DF, Landschoot RL. J Applied Microbiolgy 1973; 25: 501-502
[22] Yun JW, Lee MG, Song SK. J Ferment Bioeng 1994; 77: 159-163
[23] Van doorn W. Acta Hortic 1989; 261: 27-34
[24] Mayak S, Dilley FR. J Amer Soc Hort Sci 1976; 101: 583-585