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# Calcium and the Biological Activities of Two *Streptomyces* Species Isolated from the Rhizosphere of Soybean Plants

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ABSTRACT. Careful examination of the characteristics of both organisms revealed that we were dealing with *Streptomyces violochromogenes* and *Streptomyces glaucescens*. Calcium chloride decreased the permeability of both organisms as indicated by the low total nitrogen content of their media compared to the controls. Small doses of calcium nitrate suppressed the permeability of *S. violochromogenes* and increased that of *S. glaucescens*, whereas the larger doses were stimulatory for both organisms.

Calcium chloride increased the accumulation of nitrogen in the biomass of both organisms. Calcium nitrate had similar effects on *S. violochromogenes* only without affecting *S. glaucescens*. The stimulatory effects of calcium gradually faded with increasing the concentration. Calcium chloride increased the proteolytic activity in *S. glaucescens* media only at the larger doses whereas calcium nitrate suppressed the proteolytic activity in *S. glaucescens* media. The cellulolytic activity of both organisms was suppressed by calcium but amylases were initiated in *S. glaucescens* media. Those of *S. violochromogenes* were arrested with the lower levels of calcium but the larger doses restored or stimulated their activity.

The metabolic activities of rhizospheric microflora play an important role in the growth of plants through supplying them with the mineralization products of organic matter. They may produce proteolytinc enzymes, growth promoting or growth retarding substances for higher as well as lower plants (Heggo 1964).

The role of rhizospheric actinomycetes received little attention in the past. *Streptomyces* species are relatively good decomposers of organic matter (Alexander 1977). Kuster (1979) reported that a large number of actinomycetes, exhibits various and unusual physiological behaviour, play an important role in the degradation of organic matter. Abraham and Herr (1964) noticed that more actinomycetes were able to hydrolyse starch in the rhizosphere of corn and

soybean. Kamel (1983) reported that nitrate reduction, amylolytic and cellulolytic activities of *Streptomyces* species were higher within the rhizosphere of soybean.

Actinomycetes and bacteria can reduce nodulation by inhibiting either *Rhizobium* or root growth or by altering the root metabolism so that infection is prevented (Dart 1974). Antoun *et al.* (1978) indicated that 70% of the actinomycetes, isolated, exhibited no inhibitory effect on *Rhizobium*.

Andrew (1976) and Banath *et al.* (1966) have reported that calcium is a triggering factor for nodulation. This investigation has enhanced us to study the role of additive calcium in the growth and metabolic activities of two *Streptomyces* species, of common occurrence in the rhizosphere of soybean plants.

## Materials and Methods

Two *Streptomyces* species (symbolized SA and SB), of common occurrence in the rhizosphere of soybean plants, growing in the farms of the Agricultural Research Centre, Gizza (Kamel 1983), were used in this investigation. The isolates were maintained on glycerol - casein hydrolysate agar (Kuster and Williams 1964) and stored at 4°C.

The media and procedures used for studying the cultural, morphological and physiological properties as well as utilization of carbon sources were those described by Shirling and Gottlieb (1966). The enzymatic activities were determined by the cup plate method (Dingle *et al.* 1953).

One hundred aliquots of liquid starch - nitrate medium containing either 1, 2, 3, 4, or $5 \times 10^{-3}$M of calcium chloride or calcium nitrate, were inoculated with 1 ml of a dense standard spore suspension of either of the *Streptomyces* species. All flasks, together with their controls, were incubated, at 28°C, for 7 days after which the biomass was filtered off and the filtrates were cleared by centrifugation at 3000 rpm, for 15 min. The pH of the media was readjusted to 7 before completion of the volume to the original 100 ml. Enzymatic activity and nitrogen components of such filtrates were assayed while the biomass was analysed for its nitrogen content only. Under all conditions, 5 replicates were used for each determination. The procedures for nitrogen analysis, mentioned by Naguib (1969), were followed in this investigation.

## Results and Discussion

### *1. Taxonomic Studies*

On most media, isolate SA formed abundant aerial mycelia which developed into flexious chains of spores (Section Rectiflexibilis) but few spirals with one or two turns were also observed [Plate 1]. The spores were oval to cylindrical with a smooth surface [Plate 2].

**Plate 1.** Micromorphology of Spore Chains of Isolate SA

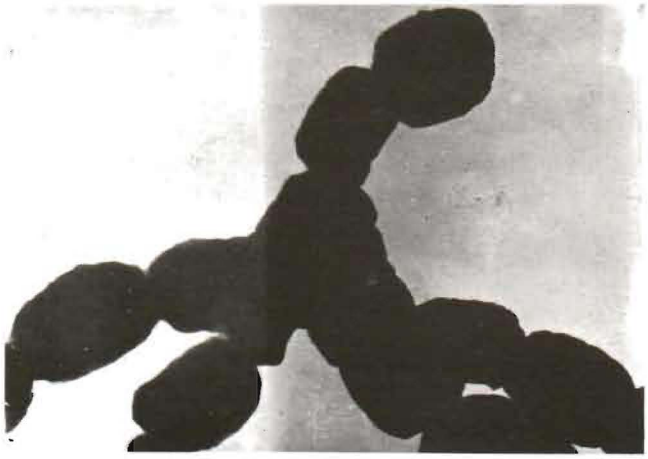

**Plate 2.** Scanning Electronmicrograph of Spores of Isolate SA

This isolate produced redish brown or grayish yellowish pink aerial mycelium on most media used (Table 1). The reverse side of the colony was redish brown, grayish red or grayish yellow. Yellow or pink pH sensitive pigment was produced in the media, changing from pale pink to yellow with acidification.

**Table 1.** Cultural characteristics if *streptomyces* isolates, SA and SB, grown for two weeks on various media

| Media | Isolate SA | | | | | Isolate SB | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Growth** | **Aerial mycelium** | **Colour of colony** | **Reverse colour** | **Soluble pigments** | **Growth** | **Aerial mycelium** | **Colour of colony** | **Reverse colour** | **Soluble pigments** |
| Inorganic salts Starch agar (I S $P_4$) | Excellent | Good | Reddish brown | Redish brown | Pale orange | Good | Abundant | Grayish green | Grayish red | None |
| Glycerol-asparangine agar (I S $P_5$) | Moderate | Moderate | Grayish yellow | Grayish red | Yellow | Moderate | Poor | Green | Reddish brown | None |
| Oat meal agar (I S $P_8$) | Good | Poor | Grayish brown | Grayish yellow | Pale | Good | Good | Grayish green | Grayish brown | None |
| Yeast-malt agar (I S $P_2$) | Moderate pink | Moderate | Grayish pink | Redish brown | Pale yellow | Moderate | Moderate | Green | Yellowish brown | None |

The temperature range for growth was from 15-40°C with an optimum at 28°C. Melanin production was positive on tyrosine - yeast extract; peptone-yeast extract - iron agar and tryptone - yeast extract broth. All tested carbon sources were utilized by isolate SA (Table 2).

**Table 2.** Utilization of carbon sources by *Streptomyces* strain (SA) and *Streptomyces* strain (SB)

| Carbon source | *Streptomyces* (SA) | *Streptomyces* (SB) |
|---|---|---|
| D - glucose | + | + |
| L - rhamnose | + | + |
| D - xylose | + | + |
| D - mannose | + | + |
| L - inositol | + | + |
| Fructose | + | ± |
| Sucrose | + | ± |
| Raffinose | + | ± |

Careful examination of the characteristics of isolate SA with the known red species, described by Waksman and Lechevalier (1961), Shirling and Gottlieb (1968a,b; 1969; 1972) and other recent literature showed that this isolate very closely resembled *S. violochromogenes* (Krasilnikov 1960; I S P 5207) in most, if not all, its characteristics and thus it was identified as such.

Mature spores of strain SB consisted of 10-50 spores and formed spirals (section Spirales) [Plate 2]. The spores are oblong to elongated with spiny or hairy surface [Plate 4]. The aerial mycelium was green or grayish green in colour, on most media. The reverse side of the colony was grayish brown, yellowish brown in colour, that turned to redish brown at later stages. The organism did not produce any diffusible pigments (Table 1).

The temperature range for growth was from 15-40°C, with an optimum at 28°C. Melanin production was positive on tyrosine agar; peptone - yeast extract - iron agar and on tryptone - yeast extract broth. All tested carbon sources were utilized (Table 2) except fructose, sucrose and raffinose that were feebly utilized by the strain.

Comparison of the characteristics of isolate SB with those for species of the green group of *Streptomyces* (Shirling and Gottlieb 1968a,b; 1969; 1972) revealed a very close similarity of the experimental organism with *Streptomyces glaucescens*

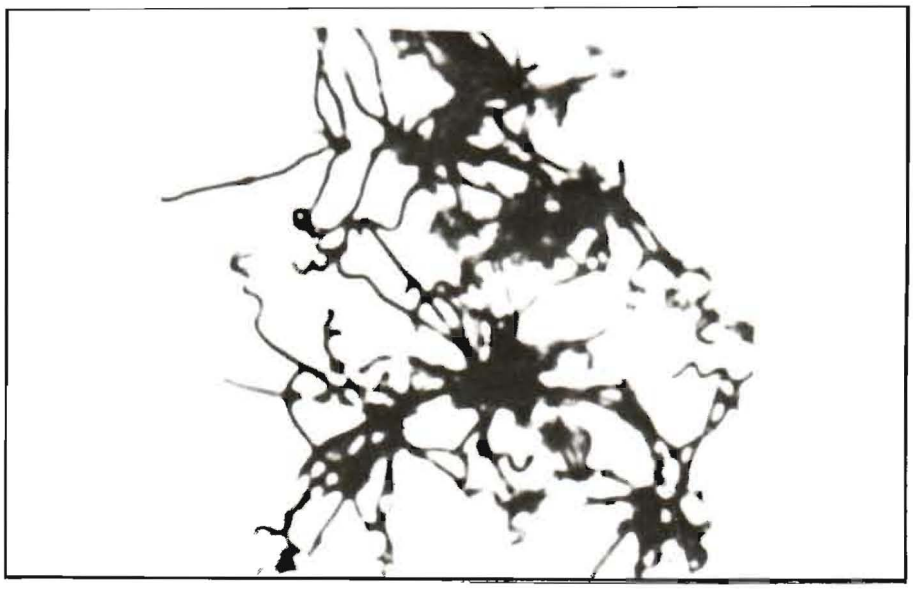

**Plate 3.** Micromorphology of Spore Chains of Isolate SB

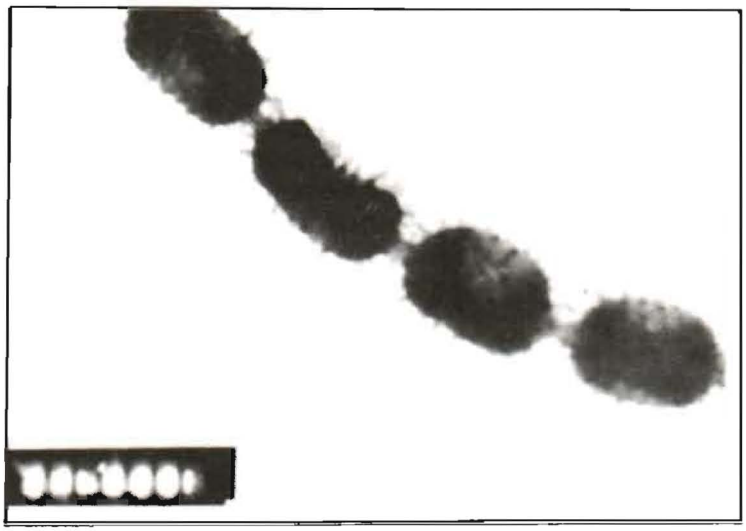

**Plate 4.** Scanning Electronmicrograph of Spores of Isolate SB

(Pridham *et al.* 1958; I S P 5155). The only difference was found in the utilization of D-fructose. Strain 5155 could utilize this sugar but isolate SB could not. This only difference was considered a strain characteristic and insufficient to regard our isolate as a new species.

***2. Metabolic Activities***

Figure 1 shows that calcium chloride suppressed the permeability of both organisms; almost to the same extent irrespective of concentration and/or species. This is obvious from the equally lower total nitrogen, secreted in the media of both organisms. Small doses of calcium nitrate suppressed the permeability of *S. violochromogenes* cells but increased that of *S. glaucescens* whereas larger doses were stimulatory to the permeability in both organisms. Increased cell permeability of *S. glaucescens* was hardly affected by calcium nitrate concentration as could be observed from the almost constant amount of nitrogen secreted in the media.

It may be mentioned that Bernheim (1970), working with *Pseudomonas aeruginosa,* reported that calcium inhibited or even arrested leakage of intracellular contents and lowered the diffusion gradient of salts across the cell membranes. It prevented the exposure of the sulphydryl groups by a cross - likage action.

It is interesting to note that the larger total nitrogen content of the media of either organisms, in presence of calcium nitrate, was the result of larger retention of nitrate nitrogen accompanied by a high increase in ammonia and other soluble nitrogen.

These observations indicate that such high nitrogen content was not the result of rapid oozing of nitrogen metabolites but due to hindered absorption; in other words, decreased permeability. The increase in ammonia and other soluble nitrogen with minor differences in amino and peptide further suggest that calcium nitrate suppressed whereas calcium chloride insignificantly affected the incorporation of nitrate nitrogen into amino acids and peptides that were secreted into the media.

Eaton and Ensign (1980) and Salas *et al.* (1985) proved that calcium is an essential triggering factor for spore germination and growth of several *Actinomyces* species. Bukau *et al.* (1985) proved that calcium treatment rendered the outer membrane of *E. coli* reversibly permeable to macromolecules. Eaton and Ensign (1980) showed that 0.5 mM calcium chloride was optimum for spore germination; larger doses were inhibitory. 2.5 mM concentration almost completely suppressed germination.

The lower total nitrogen secretion coupled with smaller amounts of remaining nitrate nitrogen in the media of calcium chloride - treated organisms indicates

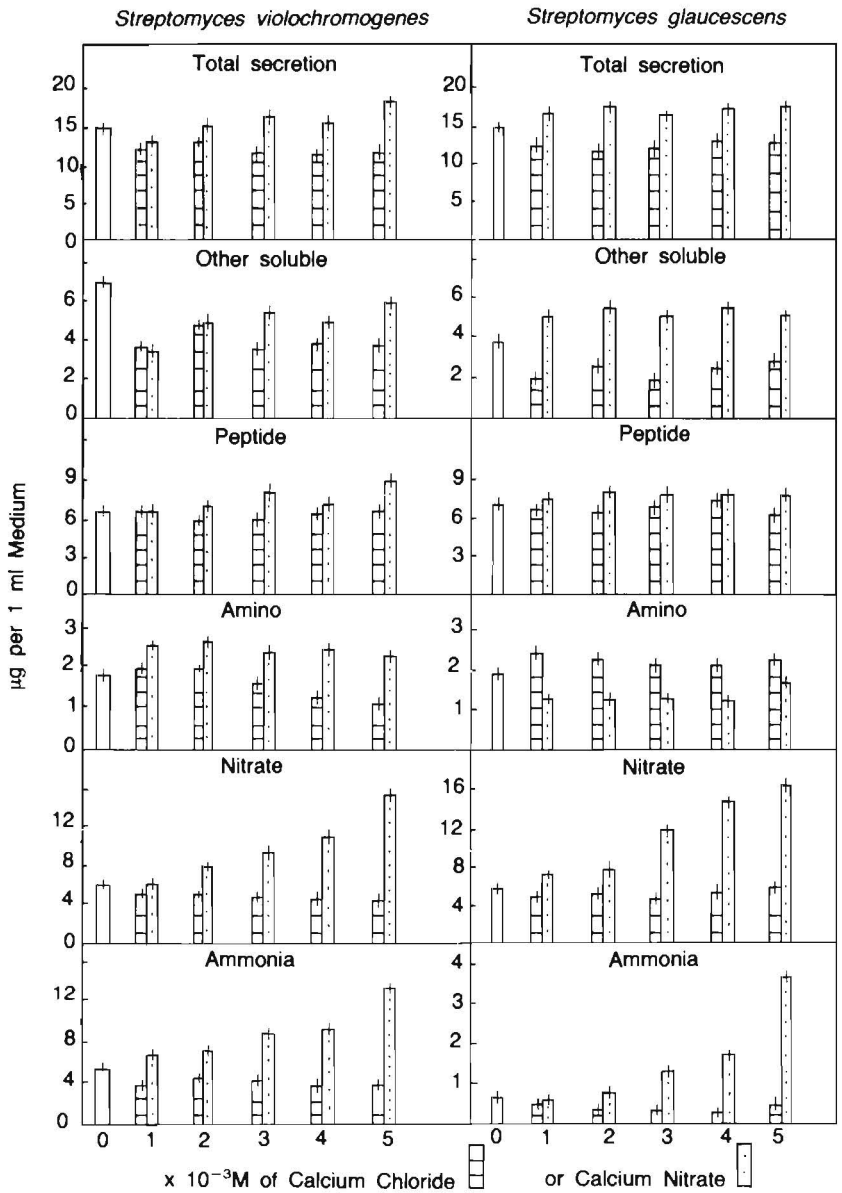


**Fig. 1.** Effect of various concentrations of calcium chloride and calcium nitrate on the nitrogen components secreted by 7-day old cultures of *Streptomyces violochromogenes* and *Streptomyces glaucescens.*

better retention and increased metabolic activity within the biomass of both organisms. This is quite obvious from the relatively larger amounts of accumulated nitrogen in the biomass of both organisms that was almost unaffected (*S. violochromogenes*) or slightly suppressed (*s. glaucescens*) with increased concentration of the salt.

On the other hand, although addition of calcium nitrate meant an increase in the nitrate supply to the growing mycelia, yet there was no appreciable effect on nitrogen accumulation within *S. glaucescens* biomass but a noticeable increase was observed in *S. violochromogenes* biomass that declined with increasing the concentration of the salts (Table 3).

**Table 3.** Effect of various concentrations of calcium chloride and calcium nitrate of the nitrogen content of 7-day old biomass of *Streptomyces violochromogens* (SA) and *Streptomyces glaucescens* (SB)

(mg per Biomass)

| Treatment | | S A | S B |
|---|---|---|---|
| Control | | 2.45 ± 0.08 | 2.81 ± 0.12 |
| 1 mM | Calcium chloride | 6.33 ± 0.23 | 6.05 ± 0.21 |
| | Calcium nitrate | 6.14 ± 0.22 | 2.87 ± 0.11 |
| 2 mM | Calcium chloride | 6.07 ± 0.23 | 5.46 ± 0.19 |
| | Calcium nitrate | 4.99 ± 0.18 | 2.42 ± 0.09 |
| 3 mM | Calcium chloride | 7.43 ± 0.26 | 5.65 ± 0.21 |
| | Calcium nitrate | 5.26 ± 0.20 | 1.91 ± 0.08 |
| 4 mM | Calcium chloride | 7.50 ± 0.27 | 4.76 ± 0.17 |
| | Calcium nitrate | 4.78 ± 0.17 | 2.11 ± 0.09 |
| 5 mM | Calcium chloride | 7.18 ± 0.26 | 5.04 ± 0.20 |
| | Calcium nitrate | 3.16 ± 0.14 | 1.97 ± 0.10 |

These observations point to the significant role of the accompanying ion. Nitrate, being an essential ion, seemed to facilitate the absorption of more calcium which, in turn, caused some toxicity that was apparent through excess release of nitrogen into the media and lower retention in the biomass. It seems that *S. glaucescens* was less tolerant to calcium toxicity than *S. violochromogenes*. This is

obvious from the larger amounts of secreted nitrogen coupled with lower nitrogen content of biomass than the controls particularly with application of the larger doses of the salt.

The larger absorption of nitrate nitrogen, in presence of large doses of calcium nitrate, might have acted as a structural analogue of the true co-repressors for the enzymes concerned with nitrogen metabolism. Whalen and Berg (1984) showed that excess L-alanine or L-valine or L-α-aminobutylic acid repressed avt A, which encodes transaminase C (alanine-valine transaminase). They termed such repression by structural analogues of true co-repressors "gratiuitous repression". Still the inhibitory effects of calcium nitrate might be explained on the basis of catabolic repression as indicated by Zimmermann *et al.* (1977). In the meantime, Mas *et al.* (1980) found that calcium chloride (up to 50 mM) initiated rapid mating in *Kluyveromyces lactis*. Similar effects were reported by Miyakawa *et al.* (1985) working with *Rhodospiridium toruloides*. Shalaby (1986) reported that calcium nitrate soaking was a better stimulant to growth and metabolsim of soybeans than calcium chloride.

The results in Fig. 2 add further evidence to the selective action of calcium ions dependent on species and/or accompanying ion. Here both tested organisms possessed equally active proteolytic and cellulolytic potentialities but extracellular amylases were lacking from *S. glaucescens* media. Calcium chloride increased the proteolytic activity in *S. violochromogenes* media; a phenomenon that was only apparent, at the largest dose, when supplemented to *S. glaucescens* media. Calcium nitrate slightly, if at all, increased *S. violochromogenes* extracellular proteinases but suppressed the proteinase activity in *S. glaucesens* media, particularly at a higher salt levels. This lends further support to the repressing or co-repressing effect of the accompanying nitrate ion.

Furthermore, calcium inhibited both cellulases and amylases activities. This is obvious from the lower activity of the former enzyme of both organisms and the complete cessation of amylase production in *S. violochromogenes* media except at the larger doses of calcium nitrate which restored or even increased the productivity of the enzyme. On the other hand, calcium nitrate induced appreciable amylolytic activity in *S. glaucescens* media that were short of such enzyme in absence of calcium chloride.

This last observation might suggest that the original calcium content of *S. glaucescens* was not enough to initiate amylolytic activity in the media of the control samples, but the nitrate (as the accompanying ion) helped in the absorption of adequate calcium ions to trigger the extra-cellular amylase production by such organism.

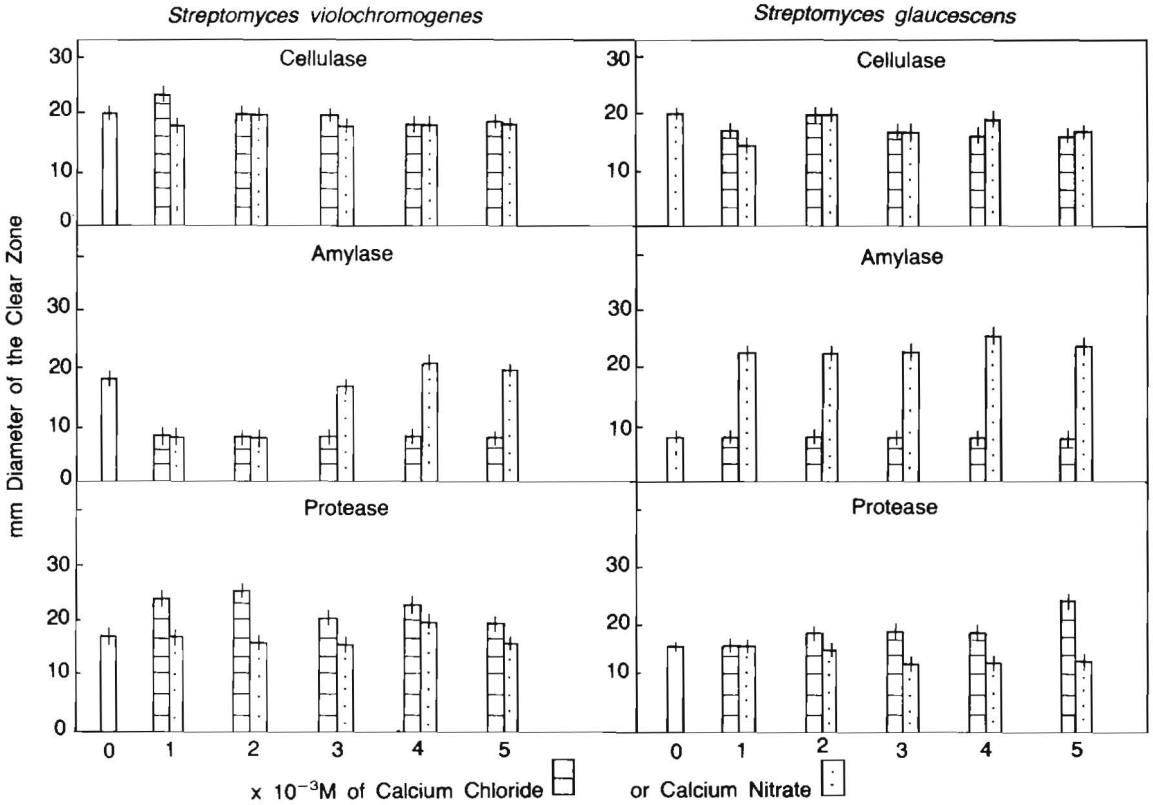


**Fig. 2.** Effect of various concentrations of calcium chloride and calcium nitrate on the extracellular hydrolases secreted by 7-day old cultures of *Streptomyces violochromogenes* and *Streptomyces glaucescens*.

Gordon *et al.* (1973) showed that calcium contributed much in stabilization of water permeability of the plasmalemma. It participated in the mechanism of opening and closure of the ion channels upon a shift of membrane potential. O'Sullivan and Noda (1968) reported that although calcium ions were poor activators of adenylate kinase in the direction of formation of A T P yet it was almost equally effctive as manganese in the direction of A T P utilization. Calcium is known to have a structural, more than catalytic, role in α-amylase activity (Hsui *et al.* 1964 and Eichhorn 1976). Calcium is required for the synthesis of proteinases of *Sarcina* strain (Coccus P) in order to stabilize the active structure of the enzyme molecule (Bissell *et al.* 1971). Maceda *et al.* (1974) noticed that calcium highly activated protease production but Andreeva *et al.* (1973) proved its non-significant effect. Taha *et al.* (1968) and Volkova *et al.* (1977) proved that calcium stimulated dextrinogenase and α-amylase production.

From the above discussion one may reach the conclusion that both *Streptomyces* species seemed to require calcium for better nitrogen assimilation and activation of their extracellular hydrolases, yet the accompanying anion and/or species seemed to verify such effects. In *S. violochromogenes,* the increased nitrogen metabolism seemed in different to the accompanying ion whereas in *Glaucescens,* the chloride ion was a better stimulant than the nitrate; the latter was even an inhibitor.

In both species, the two calcium salts suppressed the cellulase activity. The nitrate salt stimulated whereas the chloride salt inhibited the amylase. In the meantime, the chloride salt stimulated and the nitrate salt seemed without effect on the proteases.

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# تـأثير الكـالسيوم عـلى النشاط البيـولوجي لنـوعين من جنس الاستربتوميسس معزولين من حول جذور فول الصويا

زينات كامل محمد و ماري صبحي خليل و أحمد مصطفى شلبي

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اثبتت الدراسات التصنيفية للصفات المورفولوجية والفسيـولوجيـة أن هذين النـوعين المعـزولين من حـول جذور فـول الصويـا هما اسـتربتـوميسس فيـولـوكـرومـوجينـز، استربتوميسس جلوسيساتس.

أظهرت النتائج أن الإفراز النـتروجيني يقل في وجـود كلوريد الكـالسيوم وذلـك بنسبـة ثابتـة تقريبـاً مهما اختلف تـركيز الملح أو نـوع الكائن المستخـدم، أمـا نـترات الكـالسيوم فقـد أنقصت الإفراز في حـالـة الفيـولـوكـرومـوجينـز وحفـزتـه في حـالـة الجلوسياتس، أما التركيزات العالية من الملح فقـد زادت من الإفراز النـتروجيني لكلا النوعين حيث ازداد تراكم النتروجـين في الكتلة الحية لكـلا نوعي السـتربتوميسس في وجـود كلوريد الكـالسيوم وازداد الـتراكم نوعـاً (فيـولـوكـرومـوجينـز) ونقص قليـلاً (جلوسياتس) بزيادة تركيز الملح. أما نترات الكالسيوم فلم تؤثر على تراكم النتروجين في الكتلة الحية لنوع الجلوسيـاتس ولكن حفزت الـتراكم في نوع الفيـولوكـروموجينـز بنسبة نقصت تدريجياً إلى مستوى العينات غير المعاملة بزيادة تركيز الملح.

كذلك ازداد نشاط بروتيـزات ستربتـوميسس فيولـوكرومـوجينيز بنسبـة أكبر عن ستربتوميسس جلوسياتس في وجود كلوريد الكالسيوم، أما نترات الكالسيوم فلم تؤثر على بروتيزات الكائن الأول ولكنها أوقفت النشاط بالنسبة للكائن الثاني أمـا إنزيمـات تحليل السليلوز فقد نقص نشـاطها بـوضوح في وجـود ملحى الكالسيـوم خاصـة مع الكائن الثاني، أما بالنسبة للأميلاز فقد توقف نشاطها بالكامل في محاليل التغذية لنوع فيولوكرموجينز ما عدا في التركيزات العالية خاصـة نترات الكـالسيوم، أمـا بالنسبـة لنوع جلوسياتس فقد ساهم الكالسيوم في تخليق هذا الإنزيم الـذي كان معـدوماً من المنابت في غياب هذا العنصر.