STUDY OF GIBBERELLIC ACID PRODUCTION BY SOLID STATE FERMENTATION USING *Fusarium moniliforme* SHELDON

Rakesh Kumar R. Panchal1* and Piyushbhai V. Desai2

1Department of Microbiology, M. B. Patel science college, Anand, India
2Department of Biosciences, Veer Narmad South Gujarat University, Surat, India

*Corresponding author’s email: panchalrce@yahoo.com

Abstract

Gibberellic acid production using *Fusarium moniliforme*, isolated from wilted sugarcane plant has been investigated by solid state fermentation (SSF). The gibberellic acid production of 154 \( \mu \text{g/gm} \) was obtained on commercial wheat bran (CWB) mineral salt acid bed in 500 ml flasks after 168 h incubation. The gibberellic acid production rate was about 0.6 to 0.9 \( \mu \text{g/gm/hr} \) during 96 to 168 h. Different carbon sources namely sucrose, lactose, maltose, soluble starch, glycerol, wheat flour and maize flour were tested as an additional substrate along with CWB at the concentration of 25% w/w or v/w base to observe its effects on gibberellic acid production. Soluble starch has been proved the best additional carbon source for gibberellic acid production, which yielded 1160 \( \mu \text{g/gm} \) after 168 h. Similarly, various nitrogen sources namely \( \text{NH}_4\text{Cl}, \text{NH}_4\text{NO}_3, (\text{NH}_4\text{})_2\text{SO}_4, (\text{NH}_4\text{})_2\text{MoO}_4 \) and urea were tested as an additional substrate at the concentration of 0.07% w/w of CWB. Urea was proved as the best nitrogen source which yielded 532 \( \mu \text{g/gm} \) after 168 h incubation. We have observed about 7.5-fold and 3.5-fold increase in gibberellic acid production upon addition of soluble starch and urea respectively, in CWB using *Fusarium moniliforme*.

Keywords: *Fusarium moniliforme*; Gibberellic acid; Wheat bran; Maize flour; Dry moldy bran.

Introduction

A group of diterpenoid acids termed as Gibberellic acids (GAs) are largely produced by *Fusarium moniliforme* which is earlier known as *Gibberella fujicuroi*, and functions as growth regulators of plants. GAs influences a wide range of development processes in plants which includes dormancy, germination, stem elongation, sex expression, flowering, induction of enzymes and leaf and fruit senescence. The origin of research in to gibberellins can be traced to Japanese plant pathologists who were investigating the causes of the ‘bakanae’ (foolish seedling) disease which seriously lowered the yield of rice crops in Japan, Taiwan and throughout the Asian continent (Kurosawa, 1926).

The first paper on the cause of bakanae was published in 1898 by Shotaro Hori who demonstrated that the symptoms were induced by infection with a fungus belonging to the genes *Fusarium*, probably *Fusarium heterosporium* Nees. Subsequently, Elichi Kurosawa (1926) found that culture filtrates from dried rice seedlings caused marked elongation in rice and other sub-tropical grasses. He concluded that bakanae fungus secretes a chemical that stimulates shoot elongation, inhibits chlorophyll formation and suppresses root growth.

Teijiro Yabuta initiated work on the isolation of the active component using the fungal strains provided by Kurosawa. As a result, non-crystalline solid was obtained from the culture filtrate that stimulated the growth of rice seedlings. This compound was named gibberellin by Yabuta in 1935; the first use of the term ‘gibberellin’ in the scientific literature (Yabuta, 1938).

In 1938, Yabuta and his associate Yusuke Sumiki finally succeeded in crystallizing a pale yellow solid to yield gibberellin A \( \text{(C}_{22}\text{H}_{26}\text{O}_7} \) and gibberellin-B \( \text{(C}_{19}\text{H}_{22}\text{O}_3} \) (Yabuta and Sumiki, 1938). The names were subsequently inter changed in 1941 and the original gibberellin A was found to be inactive.

In the United States, the first research on gibberellins began after the Second World War by a research unit at Camp Dietrick, Maryland. In 1950, John E. Mitchell reported optimal fermentation procedures for the fungus, as well as the effects of fungal extracts on the growth of bean (*Vicia faba*) seedlings (Mitchell & Angel, 1951). Work also began at the Northern USDA Regional Research Laboratories in Peoria, Illinois in the USA using the strain provided by
Gibberellic Acid Production by Solid State Fermentation

**Materials and Methods**

**Gibberellic Acid Production by Solid State Fermentation**

Gibberellic acid production by solid state fermentation was done using the method described by Kumar and Lonsane (1987).

**Inoculum Phase**

The inoculum was grown on Czapek Dox liquid medium by adding 10⁶ spores of *F. moniliforme* 1 ml of medium (100 ml medium in 250 ml Erlenmayer flasks) and grown on rotary shaker at 150 rpm at 30°C for 48 hours.

**Production Phase**

The production medium containing commercial wheat bran (containing about 8.5 % starch on dry weight basis) was prepared.
25 gm moist medium was charged per 500 ml Erlenmeyer flasks, eight flasks were prepared from 80 gm commercial wheat bran. All flasks were autoclaved at 121°C for one hour, cooled to room temperature and inoculated with 3.75 ml, per flask, of homogenized 48 h grown culture. All the flasks were incubated in slanting position at 28±1°C for 7 days and samples were analyzed for the GA3 level at every 24 hours. A set of seven flasks had been inoculated and every day one flask was used for analytical work.

**Extraction of GA3**

After completion of fermentation the moldy bran (MB) was dried at 40°C to obtain dry moldy bran (DMB). GA3 was extracted from DMB with ethyl acetate in three stages using 10 ml each to obtain total 30 ml extract. This 30 ml ethyl acetate extract was further evaporated to 5.0 ml and used for estimation purpose.

**Estimation of GA3 by Spectrophotometric Technique**

For estimation of GA3 the spectrophotometric method described by Berrios et. al. (2004) at 254 nm was used.

**Enrichment of Commercial Wheat Bran with Different Carbon and Nitrogen Sources**

The method described by Kumar and Lonsane (1987) and (1990) was used for the enrichment studies.

The effect of enrichment of commercial wheat bran with seven different carbon sources were studied at 25 % level (w/w or v/w) based on the weight of commercial wheat bran.

Water insoluble carbon sources viz. wheat flour and maize flour were thoroughly mixed with wheat bran before moistening. Heat sensitive carbon substrates such as sucrose, lactose and maltose were sterilized at 10 lb pressure for 15 minutes and were added to the sterilized wheat bran medium before inoculation, while the water soluble carbon substrates such as soluble starch and glycerol were dissolved in mineral salt solution and then used for moistening wheat bran.

For enrichment of nitrogen sources commercial wheat bran medium was enriched with 70 mg nitrogen from different sources per 100 gm commercial wheat bran. Desired weight of different nitrogen sources except urea were dissolved in mineral salt solution and then used for moistening wheat bran. The urea solution of desired concentration was sterilized by passing through a bacteriological filter and was added to the sterilized medium at the time of inoculation.

**Results and Discussion**

**GA3 Production by SSF on WB Medium**

The amount of GA3 produced by SSF on WB medium is shown in Table 1 and Fig. 1. For the initial 3 days the production is negligible or the amount produced is not sufficient enough to be detected by the method used the maximum GA3 production was observed on the 7th day and thereafter it remained unchanged on the 8th day. There was gradual increase in the production and the rate of production from 4th day onwards. The rate of production was maximum on the 7th day but it declined on the 8th day. Although cell biomass estimation was not carried out, from the results we could say that the culture must have grown in the log phase in the initial 72 hrs and then the growth was in the stationary phase until the end of the fermentation experiment. The production phase may thus coincide with the stationary phase.

A similar pattern of GA3 production was observed with the five strains of *F. moniliforme* studied by Kumar and Lonsane (1987). In *G. fujikuroi* isolated from rice plants the production of GA3 was initiated after 72 hrs and reached to maximum on 7th day (Kumar and Lonsane 1988).

**Table 1: Rate of GA3 productions by solid state fermentation on commercial Wheat bran medium**

| Incubation period (hours) | Rate of GA3 Production (µg/gm/h) |
|---------------------------|----------------------------------|
| 96                        | 0.625                            |
| 120                       | 0.725                            |
| 144                       | 0.819                            |
| 168                       | 0.916                            |
| 192                       | 0.791                            |

Note: 10 gm commercial wheat bran was taken / flask.

**Effect of Supplementary Carbon Sources on GA3 Production by SSF in CWB Medium**

The effect of enrichment to CWB with seven different carbon sources is shown in Fig. 2. When the GA3 production was observed on 5th, 6th and 7th day, there was remarkable improvement in the GA3 production with all the carbon sources. Soluble starch was found to be the best enrichment source. There was about a 7 to 8 fold increase in the GA3 production when CWB was enriched with soluble starch. With other carbon sources there was about 3 to 4 fold increase except lactose. The increase in GA3 production...
with lactose was very little on 5th and 6th day; however, the increase on 7th day was very high. The increase in GA₃ production may be in direct correlation with the complexity of the carbon sources. Soluble starch being polysaccharide proved to be very effective for GA₃ production. In case of disaccharides, Sucrose and maltose on hydrolyses give similar hexose sugars, supported the production equally on 5th, 6th and 7th day. Lactose, giving glucose and galactose on hydrolyses, initially gave little increase but on 7th day considerable increased in GA₃ production was observed. Natural sources like maize and wheat flour supported higher production of GA₃ but the increase in GA₃ production is less compare to the soluble starch. This may be due to the difference in structure of starch in the natural sources. Results obtained are well supported by the data obtained by Kumar and Lonsane (1987). In their studies GA₃ production by G. fujikuroi was increased when CWB was enriched with twelve different carbon sources. The effect of enrichment with sucrose showed decrease in GA₃ production in the later stages. They also studied the effect of enrichment with natural sources such as Sorghum flour, Maize flour and rice bran. The yield was reduced with rice bran because of the poor growth.

![Image](http://nepjol.info/index.php/IJASBT)

**Fig. 2:** Effect of supplementary carbon sources to CWB on production of GA₃ by SSF

**Effect of Supplementary Nitrogen Sources on GA₃ Production by SSF in CWB Medium**

Results obtained for different nitrogen compounds used as supplementary sources are shown in Table 2. It was observed that the GA₃ production with urea, which was used in the control, remains as a better source of nitrogen as compared to other ammonium sources studied.

The lower production of GA₃ with ammonium salts may be due to the decrease in pH with the utilization of NH₄⁺ ions. This may not be favorable for the growth and production of GA₃. Urea as nitrogen source also exhibit buffering activity and thus resists the change in pH during the course of its utilization. Although ammonium salts are costly compared to urea, our aim was to see the comparative level of GA₃ production. If more GA₃ production can be obtained the ultimate production cost of GA₃ may be reduced. Amongst ammonium compounds, NH₄Cl gave better production of GA₃.

Urea proved to be better source. This was also reported by Kumar and Lonsane (1990). They also studied the effect of different concentrations of urea on GA₃ production by G. fujikuroi. There was an increase in production of GA₃ with the increase in concentration of urea in the range of 10 to 70 mg %. There was decrease in the GA₃ production above 70 mg % concentration.

**Table 2: Effect of supplementary nitrogen sources to CWB on production of GA₃ by SSF.**

| Nitrogen sources | GA₃ production in CWB (µg / gm) |
|------------------|---------------------------------|
|                  | Incubation period               |
|                  | 120 hrs | 144 hrs | 168 hrs |
| NH₄Cl            | 190     | 302     | 391     |
| NH₄NO₃          | 140     | 212     | 280     |
| (NH₄)₂SO₄       | 120     | 160     | 230     |
| NH₄M0O₄         | 110     | 145     | 190     |
| Urea             | 234     | 489     | 532     |
| Without enrichment | 95     | 125     | 163     |

**Summary**

The isolated culture was tested for the production of the gibberellic acid by solid state fermentation on wheat bran mineral salt acid bed in 500 ml. flasks, where considerable amount of gibberellic acid was obtained. Different carbon sources were added as additional substrate along with CWB viz Sucrose, Lactose, Maltose, Starch (soluble), Glycerol, wheat flour, Maize flour were tested for production of gibberellic acid. Soluble starch proved to be the best additional substrate for gibberellic acid production. Similarly various nitrogen sources viz. NH₄CL, NH₄NO₃, (NH₄)₂SO₄, (NH₄)₂MoO₄ and urea were tested for its effect on gibberellic acid production, where urea proved to be the best nitrogen source.

**Acknowledgements**

We are thankful to the Head, department of biosciences, Veer Narmad South Gujarat University and Management and Principal of M.B.Patel Science College, Anand for providing all the facilities for the present work.

**References**

Berrios J, Illanes A and Aroca G (2004) Spectrophotometric method for determining gibberellic acid in fermentation broths. *Biotechnology letters* 26(1): 67-70.DOI: 10.1023/B:BILE.0000009463.39203.8b

Cross BE (1959) A revised structure for gibberellic acid. *Proc. Chem. Soc.*: 302 – 303.

This paper can be downloaded online at [http://ijasbt.org](http://ijasbt.org) & [http://nepjol.info/index.php/IJASBT](http://nepjol.info/index.php/IJASBT)
Curtis PJ and Cross BE (1954) Gibberellic acid- A new metabolite from the culture filtrate of Gibberella fujikuroi. Chem. Ind. 35: 1066.

Grove JF (1961) The gibberellins. Quart. Rev. (Chem. Soc. London). 15: 46-70.

Holbrook AA (1961) Spectrophotometricic method for detection of gibberellic acid. Advances in chemistry series. 28: 159 – 167. DOI: 10.1021/ba-1961-0028.ch018

Kumar P and Lonsane BK (1987) Gibberellic acid by solid state fermentation: consistent and improved yields. Biotechnology and Bioengineering 30(2): 267-271. DOI: 10.1002/bit.260300217

Kumar PKR (1987) Extraction of Gibberellic acid from dry moldy bran produced under solid state fermentation. Process biochem.: 139 – 143.

Kumar PKR (1987) Gibberellic acid by solid state Fermentation: Consistent and improved yields. Biotech. Bioeng. 30: 267–271. DOI: 10.1002/bit.260300217

Kumar PKR (1988) Batch and fed batch solid state fermentations: kinetics of cell growth, hydrolytic enzymes production and Gibberellic acid production. Process biochem. 23(2): 43 – 47.

Kumar PKR and Lonsane BK (1990) Solid state fermentation: physical and nutritional factors influencing gibberellic acid production. Applied microbiology and biotechnology 34(2): 145-148. DOI: 10.1007/BF00166770

Kurosawa E (1026) Experimental studies on the nature of the substance secreted by the “bakanae” fungus. Nat. Hist. Soc. Formosa 16: 213–227.

Mitchell JE and Angel CR (1951) The growth – stimulating properties of a metabolic product of Phaseolus multiflorus. Phystopah. 41: 26-27.

Qian XM, Du Preez JC and Kilian SG (1994) Factors affecting gibberellic acid production by Fusarium moniliforme in solid-state cultivation on starch. World Journal of Microbiology and Biotechnology 10(1): 93-99. DOI: 10.1007/BF00357571

Stodola FH, Raper KB, Fennell DI, Conway HF, Sohns VE et al. (1955) The microbial production of gibberellins A and X. Arch. Biochem. Biophys. 54: 240-245. DOI: 10.1016/0003-9861(55)90027-8

Tomasini A, Fajando C and Barcos G (1997) Gibberellic acid production using different solid state fermentation systems. World J. Microbial. Biotech. 13(2): 203 –206. DOI: 10.1023/A:1018545932104

Yabuta T and Sumiki Y (1938) On the crystal of gibberellin, a substance to promote plant growth. J. Agric. Chem. Soc. Japan 14: 1526.

APPENDIX

Wheat bran medium for solid state fermentation of Gibberellic acid (Kumar and Lonsane , 1987):
Commercial Wheat Bran &nbsp;&nbsp;&nbsp; 80 gm
Soluble starch &nbsp;&nbsp;&nbsp; 20 gm
Linseed Oil &nbsp;&nbsp;&nbsp; 1.0 ml
Urea &nbsp;&nbsp;&nbsp; 0.15 gm
Magnesium Sulphate &nbsp;&nbsp;&nbsp; 0.007 gm

The medium was moistened with 85 ml of mineral salts solution in 0.2 N HCl which contained the following salts.

| Salt &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &nbsp;&nbsp;&nbsp; &n...