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Prospecting Germinating Sprouts as Potential Sources for Shikimic Acid Production

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Abstract: Shikimic acid is a metabolic intermediate of aromatic amino acid biosynthesis pathway and is a commercially valuable chiral building block in a variety of synthesis reactions. Recently, the persistence of avian and swine flu viruses in many countries has increased the focus on shikimic acid, which is a key precursor in the synthesis of Tamiflu, considered the most effective treatment against these flus. However, the large scale production of this drug has been limited by low availability of the shikimic acid precursor. Presently most of the shikimic acid is sourced from the Chinese star anise shrub. Due to the long cultivation times associated with this plant and its tropical nature, this source is not enough to meet the worldwide demand of shikimic acid. Other plant sources studied have been found to be inefficient for shikimic acid isolation. This work explores the potential of germinating sprouts for commercial production of shikimic acid. Five Indian legumes namely red kidney bean (rajma), soya bean, green gram (mung), moth bean (matki) and alfalfa were investigated for their shikimic acid content at different stages of germination. Maximum acid concentration was attained at 4 days from the start of germination after which the content either reached a plateau or started to diminish. Green gram (mung) was found to yield ~ 8 mg of shikimic acid per gram of sprouts (on dry weight basis) which was highest amongst the studied sprouts. The sprouts were treated with different concentrations of glyphosate (10 mM to 40 mM) at different periods and its effect on shikimic acid accumulation was observed. The acid started accumulating from the second day onward and the content declined from fifth day of germination. Treatment with glyphosate increased the shikimic acid content from 1.5 to 3 times as compared to the untreated sprouts, with soya bean sprouts yielding a maximum of ~ 13 mg of shikimic acid per gram of sprouts (on dry weight basis).

Keywords: Shikimic acid, Tamiflu, Indian legumes, sprouts, germination, glyphosate

1. Introduction

Shikimic acid is an important intermediate compound of the shikimate pathway found in plants and microorganisms. Owing to its central position in this pathway, it is a principal precursor for the biosynthesis of aromatic amino acids, phenylalanine, tryptophan and tyrosine and other aromatic compounds [1]. *In vitro*, it is used extensively as a chiral building block for the synthesis of a number of compounds in both pharmaceutical and cosmetic industries [2]. In the recent past, the focus on shikimic acid has increased since it is the key precursor for the synthesis of Tamiflu, the only drug effective against various flu viruses. This has led to tremendous increase in the demand of shikimic acid worldwide. Currently, it is sourced principally from the seeds of the Chinese plant star anise (*Illicium verum*) known to contain about 2 – 7% of shikimic acid, which is one of the highest reported estimates in plants. However, this plant is not easily cultivable being restricted to tropical regions and reaching seed bearing stage only after six years [3]. As a result, alternatives are being explored to meet the worldwide demand of Tamiflu [4]. Many researchers have investigated the amount of shikimic acid present in different parts of various plants. Reports are also available for shikimic acid extraction from some of these plant sources, none of which have been employed on commercial scale [5,6]. Microbial fermentation for the production of shikimic acid is evolving as a promising technique for large scale production. These processes mostly involve metabolically engineered strains of bacteria namely *E. coli*, *Citrobacter freundii* and *Bacillus subtilis* [2,5,6,7,8,9]. However, the major drawbacks of these processes are long fermentation periods, dilute product streams and lengthy downstream processing. Other processes involving chemical and enzymatic syntheses are not commercially viable.

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2. Production of Shikimic Acid Using Germinating Sprouts

2.1. Theoretical Basis

In an attempt to discover alternative rich source of shikimic acid, we have investigated germinating sprouts as potential targets. Our work is based on the proposition that since germinating legumes are rich sources of proteins, several amino acids may be present at their maximum levels during germination. As shikimic acid occupies a major position in the formation of these essential aromatic amino acids, larger quantities of shikimic acid may be present during this period. Five Indian legumes namely red kidney bean (*Phaseolus vulgaris*), moth bean (*Vigna aconitifolia*), soya bean (*Glycine max*), green gram (*Vigna radiata*) and alfalfa (*Medicago saliva*) were chosen for the study. Further the effect of glyphosate, a broad spectrum herbicide and a shikimic acid pathway inhibitor was also examined on the production of shikimic acid during germination. Shikimic acid is a natural product that does not normally accumulate to high levels in plants. However, when susceptible plants are treated with glyphosate, they accumulate shikimic acid to high levels. It is possible then, to use this system to produce large quantities of shikimic acid in any plant which is susceptible to glyphosate, both in plants which do not normally accumulate shikimic acid, and in plants which may naturally accumulate shikimic acid to some level [10]. Many such studies have been published wherein effect of glyphosate on the different plants has been evaluated [11, 12].

Despite the knowledge of shikimic acid being present in plants treated with glyphosate, substantial efforts by others have failed, even though there has been a significant awareness and a long felt need for improved sources of this precursor in the wake of global pandemics of flu viruses. If this method is applied to germinating sprouts, it can be used to produce shikimic acid to industrial scale levels and could potentially alleviate the limitation of this compound in the commercial production of Tamiflu.

2.2. Methodology

Five Indian legumes namely red kidney bean (*Phaseolus vulgaris*), moth bean (*Vigna aconitifolia*), soya bean (*Glycine max*), green gram (*Vigna radiata*) and alfalfa (*Medicago saliva*) were chosen for the study. These five legumes, each weighing 12 gram (dry weight) were soaked in 10 ml of distilled water overnight. The moistened sprouts were then transferred to wet muslin cloth in a 9 cm petri plates and allowed to germinate for 6 days at ~25°C in the presence of light. The sprouts were collected, weighed and analysed for shikimic acid content from the first day till the sixth day of germination. The collected samples were ground using a blender and extracted in 20 ml of 0.1 M hydrochloric acid as per the method reported by Rangachari *et al*, 2013 [10]. The crushed samples were centrifuged at 5000g at 4°C for 30 minutes. The supernatant was subjected to second centrifugation at 10000g at room temperature for 10 minutes. After centrifugation, the non-diluted sample was analyzed according to the methods of Singh and Shaner, 1998 using a spectrometer at 380 nm [13]. The mg shikimic acid/gram of untreated sprouts was determined by comparison with the standard curve. High performance liquid chromatography (HPLC) method developed by Matallo *et al*, 2009 was also employed to confirm the shikimic acid levels in the germinating sprouts [14].

The germinating sprouts were treated with 5 ml of glyphosate solutions of concentration ranging from 10 mM to 40 mM. The sprouts were subjected to the action of glyphosate from the third to the sixth day of germination and the accumulation of shikimic acid was determined using the same method as for untreated sprouts.

2.3. Results and Discussions

As shikimic acid is a major intermediate in the biosynthesis of essential amino acids necessary for the survival of the plants, it has been found to occur in many tissues of a variety of plants in sufficiently high concentrations. Shikimic acid concentration in tissues is not constant and depends on the growth stage of the plant. It is reported that shikimic acid production is higher in stages where the metabolic process rates are high as in intensive growth of the plant [5]. As germinating seeds are in early growth phase and rich in amino acids, high rates of shikimic acid synthesis can be associated with high rates of synthesis of aromatic compounds during this period.

Based on this theory, five Indian legumes namely red kidney bean (*Phaseolus vulgaris*), moth bean (*Vigna aconitifolia*), soya bean (*Glycine max*), green gram (*Vigna radiata*) and alfalfa (*Medicago saliva*) were analysed for their shikimic acid content during germination. It was observed that shikimic acid content started increasing in these sprouts from third day onwards. The content stabilized in case of kidney bean, moth bean, green gram and alfalfa seeds whereas the concentration of shikimic acid was found to decline in case of soya bean. As the germination proceeds and the shoots starts appearing, an increase in the metabolic rate leads to a gradual deceleration of shikimic acid accumulation in case of soya bean. Figure 1 shows the comparative production of shikimic acid in different sprouts. Green gram (mung) exhibited maximum acid level (~8 mg/ gram based on dry weight of the legumes) for untreated samples.

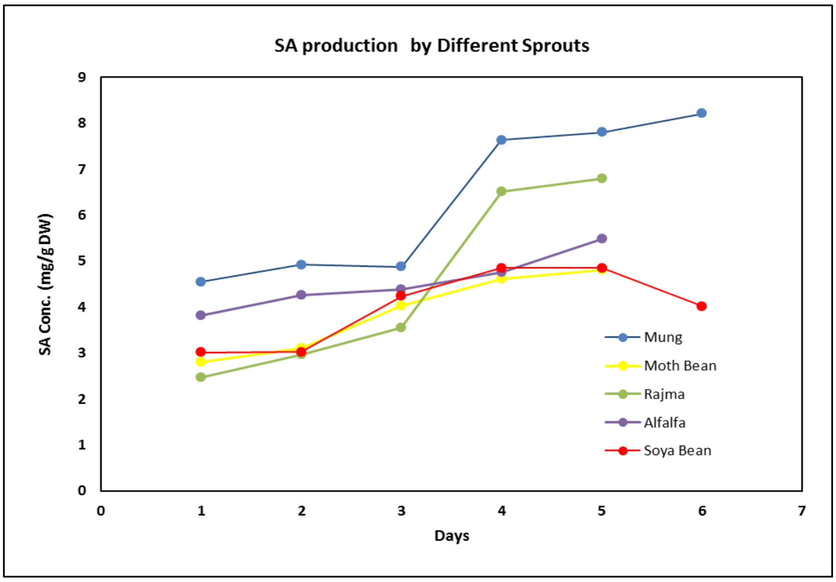

Fig. 1: Shikimic acid production by untreated sprouts

A representative chromatogram for shikimic acid analysis is shown in Figure 2.

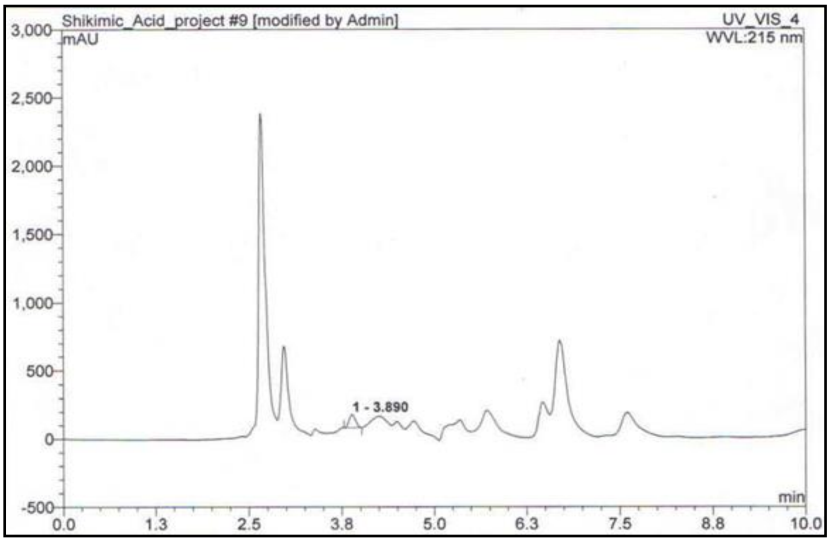

Fig. 2: Shikimic acid detection in alfalfa sprouts using HPLC

The effect of glyphosate was studied on accumulation of shikimic acid in the germinating sprouts. Glyphosate is a broad spectrum herbicide and works on the principle of inhibiting the shikimic acid pathway. It breaks the shikimate pathway by inhibiting an enzyme, 5-enolpyruvylshikimate-3-phosphate (EPSP) responsible for synthesis of amino acids and thus leads to the accumulation of shikimic acid in the tissues [15]. Different concentrations of glyphosate ranging from 10 mM to 40 mM were applied to germinating sprouts from third day onwards and it was found that 30 mM concentration gave maximum accumulation of shikimic acid in all five sprouts.

Shikimic acid accumulation started immediately after treatment with glyphosate and declined at different periods for different sprouts. Green gram (mung) and kidney bean (rajma) which showed maximum concentration of shikimic acid (~ 8 mg/ gram dry seeds and ~ 7 mg/ gram dry seeds respectively) prior to the glyphosate treatment were seen to exhibit the lowest levels after treatment. The shikimic acid levels in treated sprouts were in fact lower than the initial levels. Moth beans showed around 1.5 times increase in shikimic acid levels after contacting with glyphosate on the third day; thereafter the acid levels decreased significantly. The maximum positive effect of glyphosate treatment on shikimic acid accumulation was observed in alfalfa and soya bean sprouts which exhibited minimum levels before treatment. In these two legumes the effect of glyphosate was gradual with alfalfa showing a peak on the fourth day and soya bean on the fifth day of germination. Maximum concentration of shikimic acid was obtained in the case of soya beans (~ 13 mg/ gram dry seeds). The results of shikimic acid production in response to glyphosate application are depicted in Figure 3.

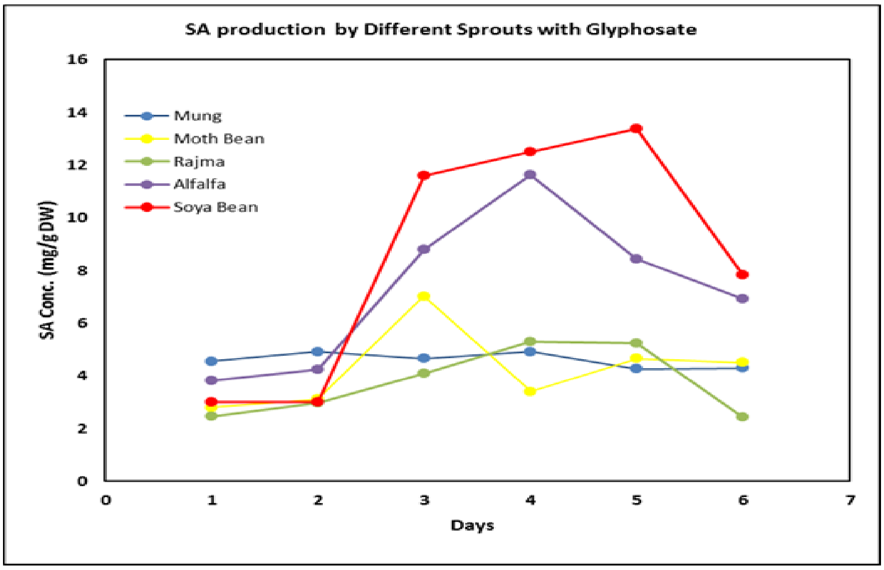

Fig. 3: Shikimic acid production by sprouts treated with 30 mM glyphosate

The different responses of germinating sprouts on shikimic acid levels on treatment with glyphosate can be due to several reasons. Some species of plants are not resistant to glyphosate and in the absence of essential amino acids immediately stop growing. This may explain the low levels of shikimic acid in green gram (mung) and kidney beans (rajma) on treatment with glyphosate. Further, inhibition of EPSP by glyphosate is non-reversible and treated plant parts are eventually starved of the essential end-products of the shikimate pathway i.e. amino acids. Since this affects new growth to a greater extent than existing parts, the symptoms of glyphosate treatment appear to be slow to develop [10]. This explains the decline of shikimic acid levels in alfalfa and soya bean sprouts after a certain period of time.

Figure 4 shows the shikimic acid content in untreated sprouts and sprouts subjected to glyphosate. Glyphosate application increased shikimic acid production by around 2 times in alfalfa and 2.8 times in soya bean sprouts. The maximum reported levels of shikimic acid has been reported to be of the order of 7.5 mg/ gram dry weight of the plant tissue found in soya bean leaves treated with glyphosate [10].

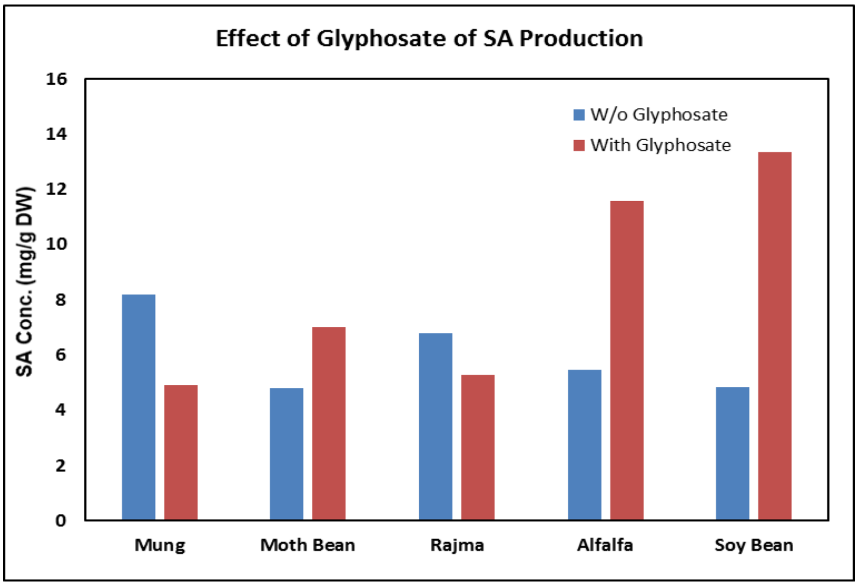

Fig. 4: Effect of glyphosate on shikimic acid yield

2.4. Conclusion

The above study shows that germinating sprouts have reasonably high concentrations of shikimic acid when compared to other plant sources. Further this content can be enhanced by treatment of the sprouts with glyphosate which is a cheap and easily available herbicide. The maximum observed yield is of the order of 13 grams per kg dry legumes which is higher than many of the reported plant species. Additionally, this method is not dependant on tropical plants for shikimic acid production and represents a simple and renewable source of shikimic acid. Shikimic acid production using the method of germinating sprouts is significantly less time consuming as compared to other processes, especially those which are fermentation based. Hence, it can be concluded that treating germinating sprouts with glyphosate can be exploited for commercial production of shikimic acid and can in a way alleviate the problem of shikimic acid scarcity.

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