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Investigation of Germicide and Growth Enhancer Effects on Bean Sprout using NMR-based Metabolomics

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Abstract Bean sprouts are often cultivated in the circumstances prevailing in the improper using of germicide and growth enhancer. The influence of ingestion those bean sprouts are unknown. The components of the bean sprouts are needed to evaluate for food safety. The extracts of the control, 0.5 g/L germicide, 1 g/L germicide, 12.5 mL/L growth enhancer and 25 mL/L growth enhancer were used to compare the components in the experiment. Nuclear Magnetic Resonance spectroscopy (NMR) was used to analyze the extracts. Statistical analysis of metabolomics showed significant changes between the control and head and the stem of the bean sprouts. Significant changes in metabolites were identified with the bean sprouts cultivated with germicide and growth enhancer by applying qualitative and quantitative analysis. Similar changes in the area of the bean sprouts were observed after treated to germicide and growth enhancer. Although treating germicide and growth enhancer showed no particular harmful metabolites changes to human, it made significant changes in the morphological and the metabolites of the bean sprouts. These changes indicate that the germicide and growth enhancer has substantially potential to influence the growth of the bean sprouts.

Keywords NMR spectroscopy, Metabolomics, Bean sprout, Multivariate statistical analysis

Introduction

Recently, the farmers are using germicide and growth enhancer to increase the yield of bean sprouts. An unauthorized agricultural pesticides are often used to cultivate the bean sprouts. Residual agricultural pesticides exceeding the acceptable limit were found in bean sprouts that consumed in the market. Growth enhancer is regarded as a growth control in most of the farms, and its use and side effect has been reported.[1] Up to now, chemicals such as an agricultural germicide and growth enhancer are widely used to grow plants in farms. However the effect of using these chemicals are yet to be evaluated. Most of the growth enhancers are known to be relatively low in toxicity,[2] but previous studies have reported that the antioxidant defense system is inhibited or malformed, and there is a growing concern about the side effects of growth enhancers.[3] The study of changes in plants components by treating germicide and growth enhancer are needed to be investigated. Moreover, the study on the effect of

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ingesting those bean sprouts has to be considered. The analysis of bean sprouts exposed to agricultural germicide and growth enhancer means a lot, especially when the interest of food safety issue is higher than ever.

Agricultural germicide that used in this study was Metalaxy-M. Metalaxyl [(R, S) methyl-N-(2-methoxyacetyl)-N-(2,6-xylyl)-dl-alaninate] is an acylamine germicides, being the most widely known. This germicides, synthesized in 1977, is widely used to control plant diseases caused by pathogens of the *Oomycota* division, in particular, against *Phytophthora infestans* and *Phytium ultimum*. And Atonik was used as a growth enhancer. Atonik is an aromatic nitro phenolic compound that stimulates plant activity without causing malformation or toxicity to the plants and accelerates the plasma streaming of the cells by increase in the endogenous auxin level.[4]

The object of this study was to identify the changes of metabolome of bean sprout and the potential influence on humans. Through this study, we are expected to suggest a guideline for food safety of the bean sprout which is a common ingredient across the world.

The cell synthesizes the protein by using DNA information, and the protein acts like a biocatalyst which is also regarded as an enzyme, control the chemical reaction in the cell.[5] These reactions are referred to metabolic process, and the metabolome is a by-product of the chemical reactions.[6] Nuclear Magnetic Resonance (NMR) spectroscopy is mostly used in the study of metabolomics.[7] Each compound can be analyzed by quantitatively and qualitatively by using the chemical shift information that the mixtures uniquely has. NMR is then introduced to identify the mixtures without separating each molecule. This method requires a large number of samples since it has relatively low sensitivity. However, minimal pre-preparation and high-reproducibility enables to increase the possibility of application.[8]

In this study, NMR spectroscopy was applied to analyze the metabolome of the bean sprouts which is widely used to analyze the structure of chemical molecules and experimental tool of the metabolomics study to find out the potential effect of using agricultural germicide and growth enhancer.

Experimental Methods

Bean sprouts cultivation- Beans of bean sprouts were soaked in distilled water, germicide 0.5 g/L and1 g/L (Metalaxyl-M, Agrotech, Seoul, Korea) and growth enhancer 12.5 mL/L and 25 mL/L (Atonik, Hankooksamgong, Seoul, Korea) for 2 hours. After soaking, beans were washed. 5 groups were cultivated in automatic cultivator for 48 hours. The automatic cultivator circulated water every 40 minutes for 30 seconds. The water was changed after 24 hours of cultivation.

Extraction of metabolites- Bean sprouts were lyophilized for 12 hours. Heads and stems of bean sprouts were separated.

4 samples of heads (0.24 g) and stems (0.06 g) in each group were grinded with mortars and pestle. Optimized Bligh and Dyer extraction method was used.[9] After the extraction, aqueous layer was lyophilized.

NMR spectroscopy- Lyophilized samples were re-dissolved in 700 µL of D2O containing 2 mM of TSP-d_4 (3-(Trimethylsilyl) propionic-2,2,3,3-d_4 acid sodium salt) and transferred to 5 mm NMR tube. 600 MHz Agilent NMR spectrometer (Agilent Technologies, Palo Alto, CA, USA) was used. A Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence was used for the reducing the signals of water and macromolecule.[10] ^{1}H NMR spectra were acquired using 9.8 µs of 90° pulse, 1.5 s of relaxation delay, 3 s of acquisition time and 13 min of total acquisition time. 128 transients were collected for each sample.

Metabolic analyses- Multivariate statistical analyses were performed using SIMCA-P+ 12.0 software (Umetrics, Umeå, Sweden). NMR spectra were binned from 0.5 ppm to 10 ppm and water peak area (4.68-4.88 ppm), ethanol peak area (1.14-1.2 ppm,

3.61-3.67 ppm) and methanol peak area (3.32-3.36 ppm) were excluded. The binning size was 0.001 ppm and the binning results were normalized to total area. Principal component analysis (PCA), Partial leasts squares discriminant analysis (PLS-DA) and orthogonal partial least squares discriminant analysis (OPLS-DA) were performed using SIMCA-P+ software. Identification and quantification of metabolites were achieved using Chenomx NMR suite 7.1 software (Chenomx Inc., Edmonton, AB, Canada).

Results

Bean sprouts cultivated with an agricultural

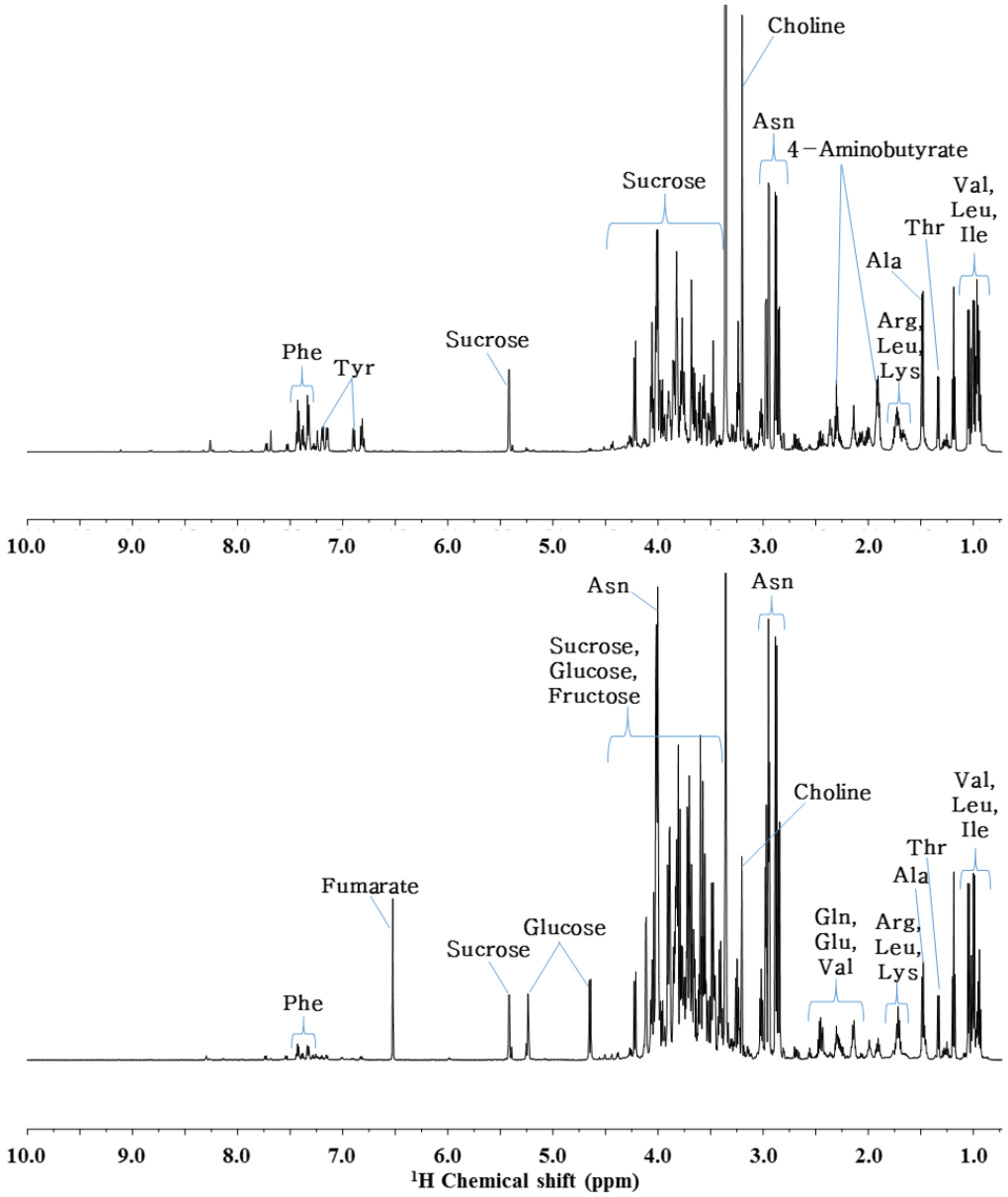

Figure 1. Representative ^{1}H NMR spectra of bean sprout head (top) and bean sprout stem (bottom). Asn: asparagine, Ala: alanine, Arg: arginine, Glu: glutamate, Gln: glutamine, Ile: isoleucine, Leu: leucine, Lys: lysine, Phe: phenylalanine, Thr: threonine, Tyr: tyrosine, Val: valine.

germicide and growth enhancer had thicker stem and lesser feeder roots compared to control. The bean sprout that treated with 1 g/L germicide and 25 mL/L growth enhancer, it had less feeder roots compared to maximum amount of allowed weight. Except for hat, there were no other particular differences. 40 samples

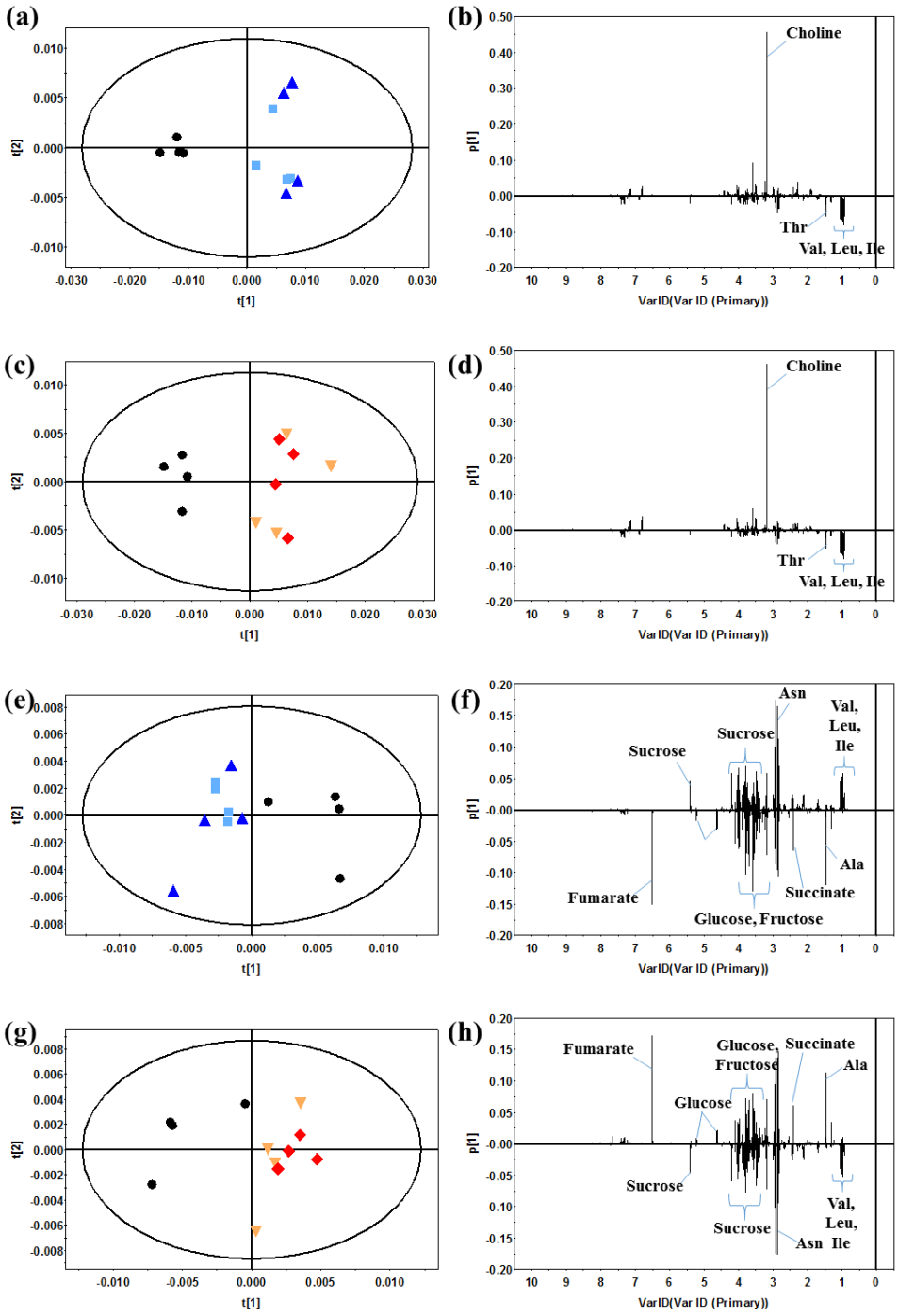

Figure 2. PCA score plots and loading plot for the NMR spectra of head of the bean sprout treated to germicide (a, b) (R^2X=81.7%, Q^2=70.4%), grow enhancer (c, d) (R^2X=87.5%, Q^2=73.2%), stem of the bean sprout treated to germicide (e, f) (R^2X=79.2%, Q^2=41.2%) and grow enhancer (g, h) (R^2X=92.8%, Q^2=68.2%). ●, Control; ■, germicide 0.5 mg/L; ▲, germicide 1 mg/L; ▼, grow enhancer 12.5 mL/L; ◆, grow enhancer 25 mL/L.

of aqueous layers were analyzed by NMR, and the spectrum of head and stem of the bean sprouts are indicated in figure 1. Also the spectra of metabolites are indicated. Several kinds of amino acids were identified significantly in the head and the stem spectrum in all the samples.

Asparagine was the dominant amino acid in the bean sprout; Choline was a primary metabolite in head; Glucose and fumarate were the significant metabolites in the stem.

For multivariate statistical analysis, spectral regions between 0.5-10 ppm were binned in segments of 0.001 ppm width and integrated the total spectral area within each bin. Bins from 1.14 to 1.2 ppm (ethanol), 3.61 to 3.67 (methanol), and 4.68 to 4.88 ppm (residual water) were excluded. Score plot was applied to NMR spectra data matrix where each spectrum is represented by a scatter plot. Score plot is the summary of the relationship among the samples. In the score plot, correlated variables will appear near each other along t [1] axis. R^2 parameter indicates the explained variation in the data and goodness of fit and Q^2 parameter represents the predictive power of the model. In this study, statistical analysis showed a significant separation in the head and the stem between the treated bean sprout and the control. Both head and stem have changed significantly after treated to an agricultural germicide and a growth enhancer, which means that NMR spectrum have also changed as these metabolites change. The main advantage of score plot is that the spectral differences are easily distinguished. In contrast, loading plot shows how the original variables contribute, to separating the metabolites along t [1] axis. Along t [1] axis, the scatter plot can be located on the right side (+) of the score plot, and up (+) in the loading plot. In the head of the bean sprout, both of score plot and loading plot showed similar result between the agricultural germicide and a growth enhancer. The result demonstrated that choline was a significant metabolite that contributed mostly to separate whereas, threonine, leucine, valine, and isoleucine contributed most to the control.

Also, the similar results were observed in the stem of the bean sprout. In the stem, fumarate, glucose, fructose, succinate, alanine and threonine contributed most to separate in the score plot. Sucrose, asparagine, valine, leucine and isoleucine contributed mostly in the control.

Table 1. Relative concentrations of head metabolites (%)

Metabolites	Control	Germicide 0.5 g/L	Germicide 1 g/L	Growth Enhancer 12.5 mL/L	Growth enhancer 25 mL/L
4-Aminobutyrate	2.5669	5.5849	4.7656	4.0595	3.7463
Alanine	5.1090	3.2417	3.1754	3.6718	3.1178
Arginine	4.7719	5.2852	6.7511	5.4543	5.9122
Asparagine	36.6038	42.1531	37.2385	39.2432	41.3098
Aspartate	2.5920	1.9097	2.0232	1.6520	1.4446
Choline	3.2593	7.1558	8.3317	8.0942	7.4808
Ethanolamine	0.4952	1.3129	1.4357	1.3552	1.2628
Fumarate	0.0485	0.1860	0.1433	0.1353	0.1851
Glucose	0.6654	0.9197	0.9062	0.9695	0.9140
Glutamate	3.0676	3.2365	4.7212	5.1642	6.7513
Glutamine	1.8158	0.8050	0.9191	0.9128	0.9406
Glycine	1.5553	1.0872	1.0969	1.1339	1.0379
Isoleucine	3.3629	1.3228	1.2494	1.1725	1.2142
Leucine	3.0139	1.1652	1.2024	1.0365	0.9725
Lysine	1.2508	0.9146	0.9767	0.9155	0.9147
Methionine	0.4554	0.4308	0.4945	0.4224	0.3606
Phenylalanine	3.2068	1.7095	1.5219	1.4811	1.4374
Proline	1.9794	1.9967	2.1768	1.9246	1.6288
Serine	7.3692	5.5164	6.1107	6.2617	5.7539
Succinate	0.1679	0.6689	0.6272	0.4896	0.5276
Sucrose	5.9332	4.8998	5.5636	6.0789	5.2484
Threonine	2.5199	1.9041	2.0016	1.9249	1.8388
Trigonelline	0.2247	0.5939	0.6838	0.6485	0.5831
Tryptophan	1.1182	1.4412	1.3258	1.4955	1.4629
Tyrosine	1.5181	0.3750	0.4400	0.2954	0.2263
Uridine	0.1190	0.2859	0.2937	0.2381	0.2166
Valine	4.1703	1.9028	1.9154	1.7912	1.6778
myo-Inositol	1.0397	1.9947	1.9088	1.9778	1.8332

Quantitative and qualitative analysis of the most contributing regions in the score plot allowed us to identify 28 metabolites from head and 26 metabolites from the stem. The weight of each sample was different. The concentration of individual metabolite

was normalized to the sum of total concentrations of the metabolite. In Table 1 and 2, the head and the stem of metabolite relative concentrations are represented, between control and the treated groups. Asparagine which is an important component of a bean sprout was contained 40% in the head and the stem.

Table 2. Relative concentrations of stem metabolites (%)

Metabolites	Control	Germicide 0.5 g/L	Germicide 1 g/L	Growth enhancer 12.5 mL/L	Growth enhancer 25 mL/L
4-Aminobutyrate	0.7805	0.6384	0.6628	0.5434	0.6170
Acetate	0.1298	0.1750	0.1754	0.1875	0.1834
Alanine	1.7303	3.7102	4.0565	3.3210	3.6804
Asparagine	44.5356	41.9622	40.6174	42.4825	41.1957
Aspartate	0.9922	0.1908	0.1714	0.1932	0.2617
Cadaverine	1.4360	1.1233	1.3884	1.2379	1.2466
Choline	0.7774	0.8500	0.8351	0.8467	0.7806
Ethanolamine	0.3615	0.3749	0.3862	0.3872	0.3972
Formate	0.0449	0.0375	0.0456	0.0391	0.0489
Fructose	15.9827	17.9708	18.6416	17.9434	17.9571
Fumarate	3.7937	6.4701	5.8818	6.0641	6.8022
Glucose	10.3165	10.7869	11.5115	11.9048	10.3536
Glutamate	0.3779	0.2876	0.3008	0.2884	0.3990
Glutamine	1.7739	0.7952	0.6793	0.6819	0.7723
Isoleucine	2.2441	1.7578	1.6784	1.6758	1.9900
Leucine	0.7740	0.6208	0.5227	0.5884	0.6872
Phenylalanine	0.7025	1.1095	1.0185	0.9273	1.1243
Proline	0.3399	0.4880	0.4588	0.4753	0.4558
Serine	3.7762	3.0280	3.0683	2.7365	3.1172
Succinate	0.1767	0.4631	0.5149	0.4719	0.5610
Sucrose	2.9445	1.1584	1.2727	1.2779	1.3178
Threonine	1.3821	1.6239	1.7447	1.6767	1.6173
Trigonelline	0.0478	0.0517	0.0491	0.0508	0.0656
Tryptophan	0.3431	0.3170	0.3188	0.2915	0.3139
Valine	3.7818	3.3774	3.2017	3.1367	3.4358
β-Alanine	0.4546	0.6315	0.7975	0.5700	0.6188

The sugar fructose, glucose showed relatively high in the stem. Alanine, aspartate, glutamine, glycine, isoleucine, leucine, lysine, phenylalanine, serine, threonine, tyrosine, and valine decreased in head relative to the controls, after treated to an agricultural germicide. In contrast, 4-aminobutyrate, arginine, asparagine, choline, ethanolamine, glutamate, succinate, trigonelline, and myo-inositol were increased in the head after the treated to a germicide.

Alanine, aspartate, glutamine, glycine, isoleucine, leucine, lysine, phenylalanine, serine, threonine, tyrosine and valine were decreased. 4-aminobutyrate, arginine, asparagine, choline, ethanolamine, glutamate, succinate, trigonelline, tryptophan, and myo-inositol were increased in the head after treated to growth enhancer.

In the stem treated to an agricultural germicide, asparagine, aspartate, glutamine, isoleucine, serine, sucrose, and valine were decreased. Alanine, fructose, glucose, phenylalanine, proline, succinate, threonine and beta-alanine were increased.

After treated to a growth enhancer, the following metabolites were observed to be depleted: asparagine, aspartate, cadaverine, glutamine, isoleucine, leucine, serine, sucrose, and valine. Alanine, fructose, fumarate, phenylalanine, threonine and β-alanine were to be increased in the stem of the bean sprout.

Discussion

There was a significant change in metabolite after treated to an agricultural germicide and a growth enhancer to a bean sprout. Similar metabolites change has been observed in the head and the stem of the bean sprout. In the head, the amino acids, alanine, aspartate, glutamine, glycine, isoleucine, leucine, lysine, phenylalanine, serine, threonine, tyrosine and valine was decreased in common. 4-aminovutyrate, arginine, asparagine, choline, ethanolamine, glutamate, succinate, trigonelline and myo-inositol were observed to be increased. Multivariate analysis also revealed that threonine, valine, leucine and isoleucine were decreased, and choline was increased after treated to an agricultural germicide and a growth enhancer. In the stem, asparagine, aspartate, glutamine, isoleucine, serine, sucrose and valine were decreased whereas alanine, fructose, phenylalanine, threonine and β-alanine were increased. Sucrose,

asparagine, valine, leucine and isoleucine were decreased, fumarate, glucose, fructose, succinate, alanine and threonine were increased in the multivariate analysis.

The treatment of Metalaxy-M and Atonik caused metabolic alterations which might change the nutrition. Choline is one of the B-complex vitamins and a major component of amphipathic lipid. It is a precursor molecule for the neurotransmitter acetylcholine, which is involved in memory and muscle control.[11] Choline can be biosynthesized from serine to ethanolamine in the body. Ethanolamine is one of the most abundant group for phospholipids and constituent of phosphatidyl ethanolamine. 4-aminobutyrate is also called gamma aminobutyrirc acid or GABA. GABA is the form of carboxyl group deprotonated and the amino group protonated and widely present in the plant. It is a neurotransmitter in the mammalian central nervous system and known to be non-toxic. It can be used as medical supplies and healthy food since it acts as hypertensive.[12] Trigonelline is isolated from beans, Trigonella foenum-graecum, and also found in sweet potato, coffee plants, sea chestnut, jellyfish, and human urine. It enables to stop the cell division at G2 of a root tip cell in beans.[13] Myo-Inositol is a component of inositol which is considered as the vitamin B complex and used to be biosynthesized of polysaccharide in the cell wall. Also, it plays an important role as to control the growth.[14]

In conclusion, the bean sprout treated with an agricultural germicide and growth enhancer showed no particular harmful metabolites changes to human. Our study demonstrated that the agricultural germicide and growth enhancer might affect the global metabolome in the bean sprouts. Because the altered metabolites could be accumulated throughout food chain further public notion might be necessary. In our study, we showed that NMR-based metabolomics might be an efficient platform to perform a rapid analysis for food such as a bean sprout.

Acknowledgements

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