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A METHOD OF EARLY SELECTION OF CUCUMBER GENOTYPES INSENSITIVE TO CHILLING BASED ON DATA MINING

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Abstract. The paper presents a procedure to identify promising, chilling-insensitive cucumber genotypes from a pool of 55 breeding lines. Selected genotypes may constitute valuable material in further selection of high-yielding cultivars in moderate climate. The approach is based on determining nitrate (NO_3^-) content, nitrate reductase activity (NRA) and chlorophyll content (Chl) in cotyledons of cucumbers grown at 12°C. These observations were then used to develop a simple algorithm, which facilitates the ordering of genotypes according to their chilling sensitivity by assigning them ranks on the basis of quartile values (from 1 to 4) of determined traits. From the examined collection, 14 least chilling-sensitive genotypes were selected, i.e. their selection for further breeding carries the lowest risk. Low chilling sensitivity of the above mentioned 14 cucumber genotypes was manifested by high Chl levels and high NRA as well as high NO_3^- contents, i.e. the sum of quartile ranks ranged from 10 to 12, at a maximum of 12. Then cluster analysis was applied to select genotypes, which possess desirable levels of tested traits. Cluster analysis showed that at the division into two and into three subsets all the 14 genotypes considered promising were found within the same cluster, when these genotypes were divided into more subsets, 13 out of the 14 best genotypes were found in one cluster. The presented method may be used to select the least chilling-sensitive cucumber genotypes also from other collections. Knowing the quartile values calculated on the basis of presented results, the rank of new genotypes characterizing their chilling sensitivity may be estimated, provided that experiments are carried out under conditions similar to those used in this study. Otherwise the application of this algorithm has to be preceded by an in-depth explorative analysis and the determination of new quartile values for the analyzed traits.

Key words: cluster analysis, chlorophyll, cotyledones, nitrates, nitrate reductase, quartile values

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INTRODUCTION

Cucumber (*Cucumis sativus* L.) is an important horticultural crop of ancient origin, which has been cultivated for over 3000 years in Egypt and Northern India, where it still exists as a wild species (*Cucumis hordwicki*). Due to its subtropical origin cucumber is a thermophilic species that tolerates poorly temperatures already slightly below 12ºC. The biggest intolerance to low temperature is found in young plants [Lynos 1973, Rab and Saltveit 1996, Saltveit and Morris 1990]. Chilling temperature is a frequent occurrence in moderate climate in early spring during germination and seedling emergence, leading to several metabolic and physiological disorders which may adversely inhibit growth and even cause failure of cucumber, resulting in limited crop yields [Saltveit and Morris 1990]. A clear symptom of chilling injury in young cucumber seedlings is the yellowing of cotyledones caused by the inhibition of chlorophyll (Chl) synthesis [Tewari and Tripathy 1998]. A lack of chlorophyll or its low content result in a situation where the organs are not capable of fulfilling their photosynthetic functions, which may lead to disturbed formation of leaves and occasionally to dying out of young plants [Lasley and Garber 1978, Bisognin et al. 2005]. Moreover, photosynthetic activity of cotyledons depends on the activity of ribuloso-1,5-bisphoshate carboxylase (Rubisco) [Moran et al. 1990]. An important factor of Rubisco protein synthesis is a sufficient nitrogen supply and high activity of enzymes participating in its assimilation [Martin et al. 2002, Feller et al. 2007, Imai et al. 2008]. The basic sources of nitrogen for cucumber are nitrate ions (NO_3^-) [Ingestd 1972]. A key role in the assimilation of this nitrogen form is played by nitrate reductase (NR EC 1.6.6.1), catalyzing the reduction of nitrates to nitrites. NR activity (NRA) is regulated by many factors at the transcription and translation levels and through the activation of the enzyme found in the tissue [Hoff et al. 1992]. Chilling temperature may result in the lowering of NRA in cotyledons through the inhibition of NO_3^- uptake and transport to aboveground parts of cucumber seedlings [Rajasekhar and Oelmüller 1987].

The aim of the study was to provide early selection of cucumber genotypes for low chilling sensitivity by measurements of NO_3^- content, NRA and Chl content in cotyledons. Exploratory analysis (data mining) of obtained results was used to evaluate and order investigated cucumber genotypes in terms of chilling sensitivity. The proposed procedure may be helpful in the selection of the most promising genotypes, especially at the first stage in a breeding programme. Existing methods, already described in literature, may be used in studies on chilling sensitivity of successive generations of cucumber [Kozak et al. 2008].

MATERIAL AND METHODS

Plant material. The experiments were performed using 55 breeding lines of cucumber (*Cucumis sativus* L.) from the Station of Vegetable Plant Breeding and Seed Production, LTD 'SPÓJNIA" Nochowo, Poland. On the basis of preliminary experiments it was established that the minimum temperature for the growth of these genotypes is 12°C. Seeds were sown to boxes filled with peat substrate and placed in a growth

chamber for 3 days at a temperature of 22°C, and next plants were transferred to a chamber at 12°C for 21 days. Throughout the entire cultivation period light intensity was 120 W · m^{-2} (TLD 30W/54 Philips fluorescent lamps) at a 10/12 h photoperiod (day/night).

The same amount of plants from each genotype was grown in a separate box. In order to study the effect of chilling on seedlings grow the unfolding of the first true leaf was recorded in plants grown in each box. Individual boxes were assigned a category of 1 or 0, respectively, depending on the fact whether in most seedlings the first true leaf developed or did not develop. Than, five seedlings, which appearance was consistent with the category of the box, were harvested. From each seedling cotyledons were collected in order to estimate NO_3^- content, NRA and Chl content. Five independent measurements were performed for each trait. The mean value from five measurements was taken as an observation.

NO_3^- content was determined by the method of Cataldo et al. [1975]. Tissue fragments (250 mg) were immersed in 10 cm^3 deionized water and incubated for 20 min at 100°C. Nitrate content in the extract was assayed after salicylic acid nitration. The concentration of the coloured product of this reaction was determined by the measurement of sample absorbance at 410 nm (spectrophotometer Specol 11, Carl Zeiss, Jena). The amount of nitrates (NO_3^-) was calculated on the basis of the standard curve and expressed in micrograms per gram of fresh weight (μg · g FW^{-1}).

NRA was determined by the *in vivo* method [Jaworski 1971]. Discs (8 mm in diameter) of cotyledon tissue (200 mg) were incubated in 0.1 M phosphate buffer pH 7.5 with 2% propanol at 30°C for 1 h in the dark. The amount of nitrite released into the medium was determined by the application of 0.01% N-(1-Naphtyl)-ethylenediamine dihydrochloride and 1% sulfanilamide in 0.2 M HCl. Optical density of the developed red colour was measured at 540 nm (UV/VIS Spectrophotometre Jasco V-530). NRA was expressed as nmoles NO_2^- · g fresh $weight^{-1}$ · h^{-1} (nmoles NO_2^- · g FW^{-1} · h^{-1}).

Chl was determined according to the procedure of Hiscox and Israelstam [1979]. Dimethyl sulfoxide (DMSO) was used for pigment extraction from cotyledons (100 mg) without maceration. Optical density of the extract was measured at 649 and 663 nm in a spectrophotometer (UV/VIS Spectrophotometr Jasco V-530). Total chlorophyll content was calculated following the modified Arnon equation [Wellburn 1994] and it was expressed in micrograms per gram of fresh weight (μg · g FW^{-1}).

Statistical analysis. A considerable variation of determined traits was found, thus it is advisable to express the observations in terms of rank values assuming that analyzed traits need to be treated equivalently. A simple practical method has been proposed to select the most promising genotypes. The method arranges genotypes according to their chilling sensitivity. The following procedure was applied:

1. For the investigated traits quartile values Q_1, Q_2 and Q_3 were calculated.
2. The set of 55 observations (means) for individual traits was divided into four quartile intervals with a non-decreasing ordering.
3. Each observation was assigned a rank consistent with the number of the quartile interval, i.e. 1, 2, 3 ,4.
4. For each genotype the sum of quartile ranks for all observations was calculated.

5. Genotypes were ordered in terms of the sum of quartile ranks from the biggest to the smallest.

According to literature data, higher values of observed traits indicate lower chilling sensitivity [Bergareche et al. 1994, Tewari and Tripathy 1998]. Thus, the higher the sum of quartile ranks, the lower the chilling sensitivity of genotypes.

In order to verify the proposed method of selecting promising genotypes, the principal component analysis (Morrison 1976) and cluster analysis with the use of Ward's method were applied [Jobson 1992]. Calculations were done for the set of real results (five measurements for each trait) and for the set of means (observations). All numerical analyses were performed using the STATISTICA 8.0 package (edition 0608c-P, *StatSoft*).

RESULTS AND DISCUSSION

Table 1 presents observations of examined traits, results of data mining (quartile values Q_1, Q_2 and Q_3 ranks) and the emergence (1) or lack of emergence (0) of a true leaf incept. It may be assumed that in the analyzed group of 55, the first 14 genotypes are definitely best in terms of chilling tolerance, i.e. their selection for further breeding carries the lowest risk (tab. 1). Mean Chl content in these genotypes falls within the two highest quartiles (3 and 4), i.e. it is found markedly above the Q_2. In all of the 14 genotypes considered the most promising, high NO_3^- levels (> Q_2) were also recorded in cotyledons, which may indicate an undisturbed uptake of minerals, i.e. low sensitivity of roots to low temperature [Rab and Saltveit 1996, Kang et al. 2005]. A high quartile position (> Q_2) was also recorded for NRA in most of the 14 promising genotypes. An exception in this respect is genotype 34, occupying a low quartile position, i.e. the second quartile. However, the mean Chl level and mean NO_3^- level in this genotype are both very high (> Q_3), i.e. fall within the fourth quartile. In our earlier studies it was found that cotyledons of the cucumber genotype exhibiting a low chilling sensitivity were also characterized by a higher activity of δ-aminolevulinic acid dehydratase (ALAD, an enzyme of chlorophyll synthesis) and higher chlorophyll content in comparison to the sensitive genotype (unpublished). Moreover, it was shown that low Chl content in cucumber cotyledons at chilling temperature is attributed to increased level of active oxygen species, which inhibit key enzymes catalyzing successive steps of Chl synthesis [Van Hasselt 1972, Aarti et al. 2006]. Increased level of active oxygen species during chilling stress also results on inhibition of NRA [Bakker and Van Hasselt 1982]. Feng et al. [2003] showed that cucumber seedlings with high activities of antioxidant enzymes and low levels of active oxygen species were characterized by lower chilling sensitivity. Thus, it may be assumed that low chilling sensitivity of the above cucumber genotypes, manifested by high Chl levels and high NRA, resulted from the functioning of an effective mechanism preventing the occurrence of oxidative stress. High values of analyzed traits resulted in an appropriate functioning of cotyledons and the formation of the first true leaf, as it was seen in the first 14 genotypes. The total sum of quartile ranks for investigated traits in these genotypes ranged from 10 to 12, at a maximum of 12 (tab. 1, column 8).

Table 1. Observations, genotype ranges for analyzed traits and the appearance of the first true leaf, NRA – nitrate reductase activity

Tabela 1. Obserwacje, rangi genotypów na podstawie analizowanych cech i występowanie pierwszego liścia, NRA – aktywność reduktazy azotanowej

Genotype Genotyp No.	Nitrates Azotany		NRA		Chlorophyll Chlorofil		Genotype range Ranga genotypu	First leaf Pierwszy liść
	mean średnio	range ranga	mean średnio	range ranga	mean średnio	range ranga		
37	4.39	4	37.74	4	0.66	4	12	1
42	4.22	4	42.89	4	0.89	4	12	1
27	4.00	4	91.62	4	0.64	3	11	1
30	3.92	4	38.73	4	0.54	3	11	1
32	3.97	4	16.61	3	0.87	4	11	1
33	3.68	4	16.08	3	0.71	4	11	1
35	3.59	4	21.60	3	0.67	4	11	1
29	3.42	3	29.64	3	0.66	4	10	1
31	3.87	4	37.15	3	0.56	3	10	1
34	3.59	4	14.98 (Q_2)	2	0.68	4	10	1
40	3.30	3	37.28 (Q_3)	3	0.67	4	10	1
43	3.20	3	24.16	3	0.81	4	10	1
47	3.20	3	46.73	4	0.62	3	10	1
53	3.09	3	58.04	4	0.52	3	10	1
3	3.29	3	12.33	2	0.70	4	9	0
26	1.77	1	44.91	4	0.97	4	9	1
36	3.73	4	11.84	2	0.52	3	9	1
48	2.55	2	100.22	4	0.65 (Q_3)	3	9	1
49	1.84	1	40.74	4	0.80	4	9	1
1	3.81	4	7.49	2	0.35	2	8	0
8	0.31	1	44.45	4	0.53	3	8	0
11	1.96	1	71.04	4	0.65	3	8	0
28	3.23	3	28.35	3	0.39	2	8	1
38	2.92	2	21.28	3	0.65	3	8	1
39	3.04	2	13.66	2	0.76	4	8	1
44	2.62	2	34.20	3	0.52	3	8	1
45	3.33	3	23.58	3	0.47	2	8	1
46	2.84	2	91.01	4	0.46	2	8	1
5	2.91	2	7.32	2	0.58	3	7	0
7	2.35	1	38.66	4	0.36	2	7	0
16	3.91	4	3.64	1	0.32	2	7	0
41	3.30	3	4.46	1	0.55	3	7	0
55	3.12	3	12.47	2	0.38	2	7	1
2	3.55 (Q_3)	3	3.96	1	0.48 (Q_2)	2	6	0
14	2.01	1	9.90	2	0.62	3	6	0
20	2.77	2	6.04	2	0.39	2	6	0
23	4.19	4	4.88	1	0.05	1	6	0
25	3.50	3	13.38	2	0.01	1	6	0
50	2.64	2	18.27	3	0.31	1	6	1
51	2.64	2	25.63	3	0.24	1	6	1
54	2.87	2	11.11	2	0.40	2	6	1
6	3.42	3	3.43	1	0.31	1	5	0
13	2.99	2	6.90	2	0.19	1	5	0
15	2.98	2	2.10	1	0.44	2	5	0
17	2.16	1	5.66	2	0.34	2	5	0
21	2.41	1	7.76	2	0.44	2	5	0
22	3.18	3	3.51	1	0.20	1	5	0
52	2.48 (Q_1)	1	15.41	3	0.32 (Q_1)	1	5	1
4	3.06 (Q_2)	2	4.20	1	0.20	1	4	0
9	0.27	1	3.25	1	0.39	2	4	0
24	2.91	2	3.27	1	0.01	1	4	0
10	0.28	1	5.27	1	0.23	1	3	0
12	0.33	1	5.40 (Q_1)	1	0.18	1	3	0
18	1.49	1	0.13	1	0.19	1	3	0
19	2.00	1	2.74	1	0.13	1	3	0

The selection of genotypes, which sum of ranks ranges from 8 to 9, i.e. constitutes 66% and 75% maximum sum of ranks, seems less promising for further breeding. Four out of these genotype groups (1, 3, 8, 11) did not have the first true leaf incepts, i.e. exhibited low vitality. It seems that a lack of true leaf incepts in these genotypes was connected to very low levels of one of the analyzed traits. Genotypes 8 and 11 were characterized by a very low NO_3^- level (< Q_1), genotype 3 by low NRA (< Q_2), while genotype 1 by low NRA (< Q_2) and chlorophyll content (< Q_2) (Tables 1). In turn, true leaf incepts had genotypes 26, 44 and 49 exhibited low NO_3^- levels (< Q_1 or < Q_2), genotype 36 – low NRA (< Q_2), genotype 45 – low Chl content (< Q_2) and genotype 39 exhibited low NO_3^- level (< Q_2) as well as low NRA (< Q_2). Thus, the selection of the above mentioned genotypes for breeding is associated with a certain risk and should be verified in an experiment including the study of plants at later phases of development.

The genotypes with the sum of quartile ranks from 3 to 7 were characterized by low values of at least two traits (< Q_1 or < Q_2). Among these 27 genotypes, only in five of them (50, 51, 52, 54, 55) the unfolding of the first true leaf was observed. This group of genotypes are characterized by high chilling sensitivity and do not constitute good material for further breeding.

The obtained results confirm the significant role of nitrogen metabolism in cucumber cotyledons in the growth of young seedlings [Becker et al. 1978, Moran et al. 1990, Bergareche et al. 1994, Bisognin et al. 2005]. The results also indicate that the estimated traits may be suitable indicators in the selection of promising cucumber genotypes at the cotyledon stage.

Table 2. Cluster analysis according the first two main components, numbers of genotypes as in table 1

Tabela 2. Analiza skupień dla dwóch pierwszych składowych głównych, numery genotypów takie jak w tabeli 1

Number of clusters constructed Liczba zbudowanych skupień	Genotypes grouped in cluster 1 Genotypy zgrupowane w skupieniu 1
2 clusters 2 skupienia	3, 11, 26, 27, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 42, 43, 44, 45, 46, 47, 48, 49, 53
3 clusters 3 skupienia	3, 11, 26, 27, 29, 30, 31, 32, 33, 34, 35, 37, 38, 39, 40, 42, 43, 44, 46, 47, 48, 49, 53
4 clusters 4 skupienia	3, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 42, 43, 44, 45, 47, 53
5 clusters 5 skupień	3, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 42, 43, 47, 53
6 clusters 6 skupień	3, 29, 30, 31, 32, 33, 34, 35, 37, 38, 39, 40, 42, 43, 47, 53

According to principal component analysis the first principal component comprises 50.80% total variation in the experiment. In this component, NRA and Chl contents are equally included (41% and 47%). The second component, comprising 31.69% total variation, consists primarily of NO_3^- content (83%). Using cluster analysis the genotypes were divided into two internally homogenous groups, which are given in table 2. Cluster 1 comprises a set of 25 genotypes and contains all of the 14 genotypes with the

highest ranks (10–12), which were considered as the most promising. This cluster also contains all genotypes with ranks 9, and six out of nine genotypes with ranks 8, i.e. genotypes which in our opinion may not be considered promising. Two genotypes in this set, i.e. genotype 3 with rank 9 and genotype 11 with rank 8, did not have true leaf incepts. It seems that the affiliation of genotype 3 to cluster 1 was determined by high nitrate and chlorophyll levels, while for genotype 11 it was high chlorophyll and NRA levels. Cluster 2 comprises mostly genotypes with low levels of analyzed traits, i.e. those with ranks of 3–7 as well as three genotypes with rank 8 (1, 8 and 28). Most representatives of this group of genotypes did not have first leaf incepts. At the division into three clusters, 14 genotypes considered best, i.e. those with ranks of 10–12, were found in the same cluster of 23 elements. In turn, at the division into 4, 5 and 6 clusters, the promising genotypes belonged to one cluster of 19, 17 and 16 elements, respectively. An exception to this rule was genotype 27, which formed a new cluster with genotypes 11, 26, 46, 48 and 47. A common characteristic of this group of genotypes was their high NRA.

Cluster analysis perfomed on real measurements confirmed the division of genotypes obtained with applying cluster analysis performed on means. Thus, using the above three approaches, i.e. the principal component analysis, cluster analysis and the proposed method of selecting promising genotypes, we get the same groups of examined breeding lines. This leads to conclusion that the simple method of selecting the most promising genotypes, proposed in this study, is consistent with statistical methods. Moreover, it yields more information, as it makes it possible to order genotypes from "the best" to "the worst", and then it only depends on how many best genotypes the breeders will select. Thus, the proposed method with limited information about breeding lines may be best.

The conclusion from the study is that the proposed simple algorithm may be a good tool in the selection of promising genotypes. The method may be used to select promising genotypes from other collections also. When data from the described experiment are treated as a training sample [Larose 2005] and the quartile values are known, the rank of the new genotype may be obtained following the proposed method, provided that experiments are carried out under conditions similar to those used in this study. Otherwise the application of this algorithm has to be preceded by an in-depth explorative analysis and the determination of new quartile values for the analyzed traits.

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METODA WCZESNEJ SELEKCJI GENOTYPÓW OGÓRKA (*Cucumis sativus* L.) NIEWRAŻLIWYCH NA NISKĄ TEMPERATURĘ NA PODSTAWIE ANALIZY EKSPLORATYWNEJ DANYCH

Streszczenie. Praca przedstawia metodę selekcji genotypów ogórka charakteryzujących się małą wrażliwością na niską temperaturę. Wybrane genotypy ogórka mogą być przydatne do hodowli w warunkach klimatu umiarkowanego. W liścieniach 55 linii hodowlanych ogórka uprawianych w temperaturze 12°C oznaczano zawartość azotanów (NO_3^-), aktywność reduktazy azotanowej (NRA) oraz zawartość chlorofilu (Chl). Cechy te wykorzystano do opracowania prostego algorytmu, który umożliwił uszeregowanie genotypów zgodnie z ich wrażliwością na chłód. Na podstawie wartości kwartylowych badanych cech genotypom nadano rangi (od 1 do 4). Z badanej kolekcji wyselekcjonowano 14 genotypów o najmniejszej wrażliwości na chłód, których wybór do dalszej hodowli wiąże się z najmniejszym ryzykiem. Mała wrażliwość na niską temperaturę tych 14 genotypów przejawiała się wysokim poziomem Chl oraz wysoką aktywnością NRA i dużą zawartością (NO_3^-), czyli suma rang odpowiadających wartościom kwartylowym cech wynosiła od 10 do 12, przy maksimum 12. Przeprowadzono także analizę skupień w celu selekcji genotypów charakteryzujących się pożądanymi wartościami badanych cech. Stwierdzono, że przy podziale 55 genotypów na dwa oraz trzy podzbiory, zawsze 14 genotypów uznanych za obiecujące (niewrażliwe na chłód) znalazło się w tym samym zbiorze. Natomiast, gdy genotypy podzielono na więcej podzbiorów, to z 14 najlepszych w jednym zbiorze znalazło się 13 genotypów. Przedstawiona metoda może być wykorzystywana do selekcji genotypów niewrażliwych na chłód również z innych kolekcji. Znając wartości kwartylowe obliczane na podstawie przedstawionych wyników można oszacować rangę nowego genotypu pod warunkiem, że eksperyment będzie przeprowadzony w warunkach opisanych w prezentowanej pracy. W innym przypadku zastosowanie algorytmu musi być poprzedzone szczegółową analizą eksploratywną.

Słowa kluczowe: analiza skupień, chlorofil, liścienie, azotany, reduktaza azotanowa, wartości kwartylowe

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