METHODOLOGY FOR DIAGNOSIS OF STATE OF PLANTS USING CHLOROPHYLL FLUORESCENCE INDUCTION OF TEST PLANTS

Hanna Antonova, Volodymyr Hrusha, Anna Kedych, Oleksandra Kovyrova

Abstract: *The paper presents the authors' methodology for conducting studies of chlorophyll fluorescence induction by fluorometers developed at the Institute of Cybernetics of the National Academy of Sciences of Ukraine. The methodology based on a number of experiments conducted at the Institute. It includes the selection of the indicator plant, plant cultivation, conducting of the CFI measurements and data logging, calculation of the CFI curve parameters, graphical and statistical data analysis and application of the neural network approach. Paper will be interesting for specialists in automatization of ecological and agricultural researches that using the effect of chlorophyll fluorescence induction with application statistical and neural network approaches.*

Keywords: *chlorophyll fluorescence induction, fluorometer, data analysis and processing, neural networks.*

ITHEA Keywords: *H.4 Information systems applications.*

DOI: *https://doi.org/10.54521/ijita32-03-p05*

Introduction

The introduction of the method of chlorophyll fluorescence induction (CFI) [Kalaji, 2014], [Kalaji, 2017] into industrial plant growing requires both targeted experimental studies in the laboratory and field, as well as a number of studies in the form of observations in real conditions of forest, field, and park areas. The implementation of research in the form of observations is complicated due to a

significant number of environmental factors that are random in nature. Therefore, there is a need to use a significant number of additional sensors to register these factors, as well as to conduct chemical and biological analyzes of soil, air, parts of plant objects, etc. Therefore, at this stage of development of sensor technologies, more reliable results can be obtained by conducting targeted single-factor and multifactor experiments in the laboratory and field.

Conducting research with the help of devices for measuring CFI (such as the Floratest device [Palagin, 2017]) on test cultures consists of several stages.

Stage 1. Preliminary preparation

1. Formulate a working hypothesis. For example: Determine the quantitative and qualitative differences between the CFI values whose obtained on the control and experimental groups of plants under the influence of a heavy metal.

2. Identify the type of test plants to be studied in experiment. Indicate the Latin name. Find out the conditions of growth of the selected plant. The plant species are used in the research should meet the following requirements: illustrative of the natural zone where they located; should be located throughout the territory; have a clearly expressed quantitative and qualitative response to deviations in the properties of the environment from the environmental norm; the biology of these indicator species should be well studied. The list of plants that can serve as indicators of the impact of heavy metals on the environment is given in Table 1 [Krasnov, 2009], [Sazanova, 2012].

3. Determine the environmental factor whose impact on the plant will be studied. The reactions of plants to heavy metals [Guralchuk, 2006], [Grishko, 2014] are given in Table 2.

4. Determine the concentration of heavy metal to be applied to the soil.

It is recommended to apply heavy metals in the form of sulfuric salts. It is recommended to start from the maximum permissible concentrations for a given metal, when choosing concentrations. For example, soil contamination can be modelled by applying cadmium, zinc and copper in the form of sulfuric salts in

doses corresponding to 5 and 15 maximum permissible concentrations [Guralchuk, 2006], [Grishko, 2014] or in concentrations of 5, 10, 20 maximum permissible concentrations. The maximum permissible concentration for nickel and copper is 3.0; zinc - 23.0; lead – 20.0; cadmium – 4.0 mg/kg of soil in the form of aqueous solutions of $CdSO_4$, $CuSO_4$, $Ni(NO_3)_2$, $ZnSO_4$ and $Pb(NO_3)_2$ respectively.

For example, to model the excess copper content, choose a copper sulphate concentration of 6 g $CuSO_4$ per 1 kg of soil.

Table 1 – Indicator plants

Type	Plant
Tree species	Scots pine, spruce, birch, linden, oak, poplar
species of herbaceous and shrub layer	lingonberry, blueberry, rosemary
types of shrub layer	willow, dog rose, dwarf birch
species of herbaceous plants	Asteraceae, Rosaceae, Lamiaceae, steppe sage (Salvia stepposa), Veronica incana, V. spicata, Artemisia austriaca, A. Marschalliana, BEL W tobacco, medicinal dandelion (Taraxacum officinale), gladiolus (Gladiolus sp.), sunflower (Helianthus annuus), spinach (Spinacea oleracea), peas (Pisum sativum), beans (Phaseolus vulgaris), soya (Glycine max), quinoa (Chenopodium album), Melilotus officialis

Table 2 – Signs of excessive element content in the soil

Element	Plant reaction is observed visually
Zinc	Discoloration and death of leaf tissue, yellowing of young leaves, death of buds, red or black coloration of veins. The first signs appear on young plants.
Copper	Chlorosis of young leaves. In this case, the veins remain green. Development is inhibited, brown spots appear on the leaves and the leaves die.
Manganese	Chlorosis appears between the veins, tissue necrosis. Young leaves become bent and wrinkled.
Iron	Young leaves develop chlorosis between the veins, which remain green. Later, the leaf turns whitish or yellow.
Cobalt	Transparent areas filled with water appear along the main veins of the leaf. Tissue necrosis occurs. Later, the leaves turn brown and fall off.
Phosphorus	General yellowing of leaves of adult plants. Tissue necrosis. Necrotic spots appear on the ends and edges of old leaves.
Magnesium	Leaves slightly darken and shrink. At the last stage of growth, the

	ends of the leaves retract and die.
Potassium	In the early stages, there is a weak growth of plants, light green color of leaves. In the later stages, dry spots appear on the leaves, the leaves wither and fall off.
Chlorine	General coarsening of plants, small, dull green leaves. In some plants, purple-brown spots appear on old leaves and leaves fall off.
Nitrogen	Local damage. Chlorosis develops on the edges of the leaves and spreads between the veins. Later, brown necrosis appears, leaves curl and fall off.
Calcium	Chlorosis appears between the veins with whitish and necrotic spots that may be stained or filled with water. Leaf rosette growth, shoot death and leaf drop are sometimes observed.
Boron	Chlorosis of the leaf tips and margins occurs, spreading between the veins. Leaves turn pale yellow or whitish. Burns and necrosis are observed on the leaf margins.

5. Determine the number of variants in the experimental design and the number of plants in each of them.

It is recommended number of variants – 3-4 groups, in each group – 3-5 containers, in each container – 5-6 plants. Define one of the groups as a control group. A control group is a group of plants that are not subjected to any external influence. Subsequently, the data obtained in the experimental groups will be

compared with the data is obtained in the control group. Thus, an experimental design could be formed (example, Table 3).

Table 3 - Example of an experimental design for modelling excessive copper content

Group	Type of exposure	Containers, #
V1	without $CuSO_4$ impact, without watering	1-3
V2	control – without $CuSO_4$ impact, with watering	4-6
V3	with $CuSO_4$ impact, without watering	7-9
V4	with $CuSO_4$ impact, with watering	10-12

Stage 2. Plants cultivation

1. Sow the seeds of the selected plant in a container for seedlings. Water regularly.

2. After the seeds sprout, transplant them into containers of sufficient size with soil of the same known composition. Plant 5-6 plants in each container. Place the containers in a vegetation chamber or in a well-lit place for further growth. Water regularly. After 4-5 tiers of leaves appear, proceed to the next stage.

Stage 3. Conducting the experiment

Before starting the experiment, read the user manual for the device. The day before each series of measurements, check the charge level of the device. If the device is not charged, charge it.

Perform the first series of measurements of the CFI curves.

1. If the experiment is conducted in an open area, during sunny weather, the plants should be moved to the shade or placed under a cotton lightproof fabric with a high surface density before 60 minutes to the experiment. If the plants were grown in a climate chamber, switch off the lighting in the chamber 60 minutes before the start of the CPHi measurements. An important requirement for the chlorophyll fluorescence measurement process is to maintain the same measurement conditions. Sudden temperature changes affect the measurement results [Antonova, 2017].

2. Fix the sensor clip on the plant leaf. Take into account the tier and location of the veins on the leaf.

It is advisable to take fully formed leaves from one tier. For the positioning of the sensor for measuring the plant's CFI, choose fully formed leaves and avoid albino sectors. It is not recommended to measure chlorophyll fluorescence induction curves over a thick central vein. On small plants that have a distributed system of thin veins, the sensors can be placed randomly, as the thickness of their veins would not affect the results of measuring the CFI curves [Antonova, 2015].

3. Set the timer for dark adaptation.

If the first point is fulfilled, the time of dark adaptation could be 4-6 minutes. If the plant leaf was exposed to direct sunlight before the measurement, the dark adaptation time should be at least 20 minutes.

4. After the dark adaptation, measure the CFI curve for up to 10 s (fast CFI phase – OJIP) or the full CFI curve (from 3 to 10 min, depending on the duration of the instrument). The time should always be the same during the experiment.

5. In the experiment log, record the measurement start time, container number, measurement number in the sensor, and environmental parameters. Recommended parameters are air temperature, air humidity, soil temperature, soil moisture, soil acidity (Table 4).

6. Save the measurement data.

7. Start measuring on the next plant (go to step 2).

8. After completion of measurements, save the obtained CFI curves on a computer.

Table 4 – Example of an experiment log

Date of measurement								
Name of the plant								
Experiment number								
Container number	Group	Measurement time	Air temperature, ^{o}C	Air humidity, %	Acidity, pH	Soil moisture, units	Soil temperature, ^{o}C	Commentary

After the first series of measurements of the CFI curves, add a toxicant (e.g. heavy metal) according to the experimental design. By a series of measurements, we mean measurements of the CFI curves with the plants participating in the experiment.

In the following days, conduct 10-15 series of measurements of the CFI or until the plants in the groups grow. Measurements should be made at the same time each day.

Remarks. Conducting experiments on plants using the method of chlorophyll fluorescence induction with the help of the portable device 'Floratest' requires a certain amount of time. It consists of the following main steps: placing the device sensor on the plant leaves, conducting dark adaptation, measuring the CFI curve, recording the environmental parameters in the experiment log, transferring digital CFI data to a computer, processing the CFI curves. Estimate the required measurement time in advance when planning the experiment. The time for obtaining experimental data could be calculated using the formula:

$$t_e = \frac{\sum_{i=1}^{N}(t_{ad} + t_m + t_{pr})}{p}, \quad (1)$$

where t_e is the time of obtaining experimental data; N is the total number of measurements; t_{ad} is the time of dark adaptation of the sheet; t_{pr} is the time of preparation for the next measurement (for example, moving the sensor to another leaf); t_m is the time of measuring the CFI curve, p is the number of sensors.

Stage 4. Calculation of the CFI parameters

1. Enter the measured environmental parameters to the table in Microsoft Office Excel or other spreadsheet editor and calculate the average values.

2. Group the saved CFI curves into groups according to the experiment log.

3. Check the CFI measurement data for technical errors, and if such errors occur, exclude these curves from further consideration as misses.

4. Divide the CFI curves into groups according to the experimental design.

5. Calculate the average values of the CFI in each group for each day of the experiment using the CFiAnalyzer [Romanov, 2019] or FAnalyzer [Palagin, 2017] application.

6. Draw graphs of the CFI by group.

7. Calculate the parameters of the CFI curves using the CFiAnalyzer or FAnalyzer software. The following fluorescence parameters are calculated: F_0, F_{st}, F_m, F_v, F_v/F_m, Area, R_{fd}.

8. Calculate the average values of the fluorescence parameters.

9. Combine the parameters of chlorophyll fluorescence induction (F_0, F_m, F_{st} and F_v, F_v/F_m, Area, R_{fd}) into a table by date for further analysis.

Stage 5. Building radar chart and conducting statistical analysis

The next task after data collection is to process the results, which includes graphical and statistical analysis of the data, which allows us to identify anomalous data, patterns in the data, make certain hypotheses and test them.

1. Radar charts are a convenient way to visually analyze the difference in CFI parameters between different groups.

For the analysis using radar charts, the value of each averaged CFI parameter must be normalized by dividing each parameter value in each treatment group (F_g) by the value of the corresponding parameter in the control group (F_c). Thus, the value of the control group will be equal to one, and the values in the experimental groups indicate the change in this parameter relative to the control group:

$$\tilde{F}_{ik} = \frac{Fg_{ik}}{Fc_i}, \tag{2}$$

where $\tilde{F}_{ik}$ – the received values.

An example of radar chart is presented in fig. 1.

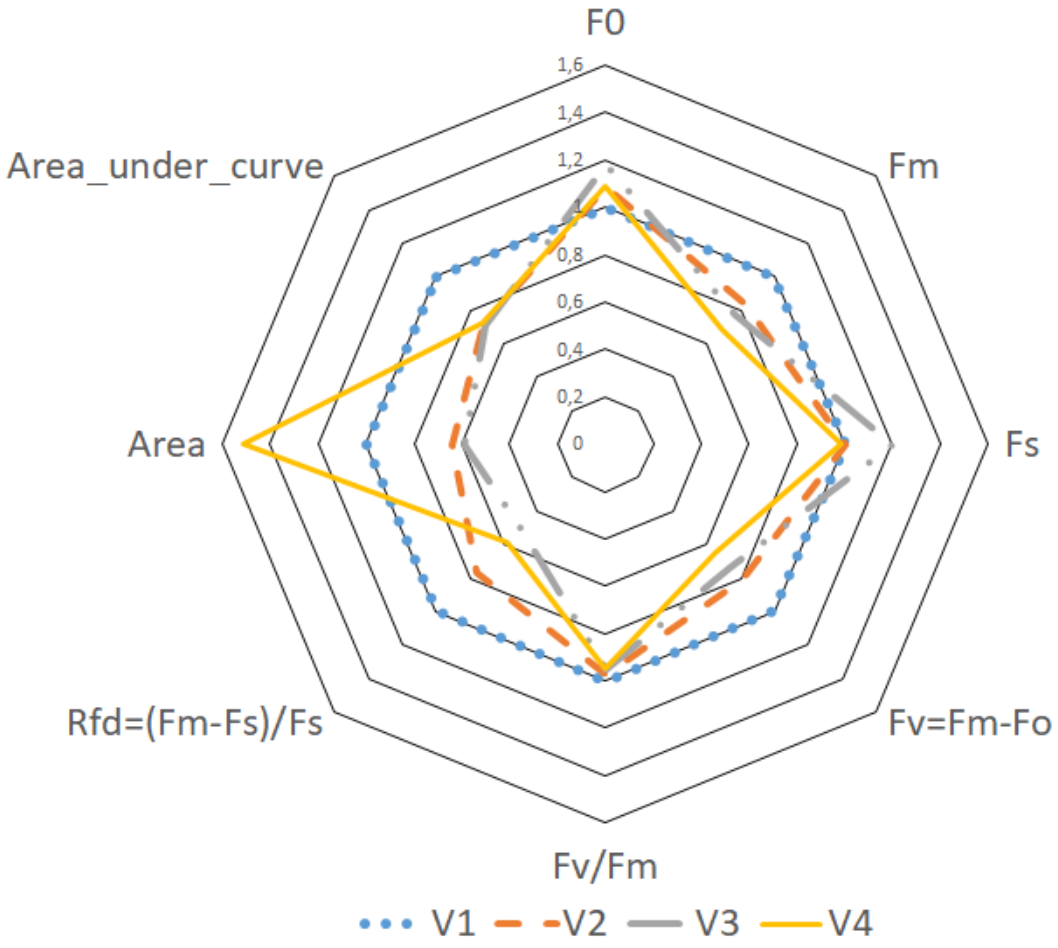

Figure 1. Changes in the parameters of CFI of experimental plants relative to the control group on the second day after toxicant treatment

2. The statistical significance of the difference between the mean values is confirmed using appropriate statistical methods, for example, Student's t-test or Duncan Multiple Range Test.

Stage 6. Statistical analysis of CFI parameters not following a normal distribution.

Statistical analysis of data is conducted using various methods depending on whether the experimental data follow a normal distribution. If the data follow a normal distribution, parametric statistical methods are used, such as Student's t-test. If the data do not follow a normal distribution, non-parametric methods are chosen. One of such methods is the Kruskal-Wallis statistical test.

This method was used for statistical analysis of the results from a controlled single-factor experiment, as presented in Table 5.

Table 5. CFI parameters from the controlled single-factor experiment

	Group 1			Group 2			Group 3		
F_0	176	176	176	112	144	112	96	112	80
F_m	2032	1776	1888	544	1024	496	240	320	224
F_v	1856	1600	1712	432	880	384	144	208	144
F_v/F_m	0.913	0.901	0.907	0.794	0.859	0.774	0.600	0.650	0.643
F_{st}	496	480	464	496	576	480	224	320	224
R_{fd}	3.097	2.700	3.069	0.097	0.778	0.033	0.071	0.000	0.000

As a result of the Shapiro-Wilk test, it was determined that most of the data do not follow a normal distribution. The Kruskal-Wallis test is conducted using the following algorithm:

1. All values from the compared samples were ordered in ascending order, regardless of their original sample.

2. Each value was assigned a rank – its position number in the ordered sequence. If values were identical, they were assigned a combined rank equal to the average of the positions they occupy.

3. The total number of observations $N = n_1 + n_2 + n_3$, is determined, where n is the size of each group. In our case, $n_1 = n_2 = n_3 = 3$, so the calculated value of $N = 9$.

4. The sum of ranks for each group, R_i, is calculated, and the average rank for each group was computed as $\overline{R_i} = R_i / n_i$. In total, three values of $\overline{R_i}$ were determined.

5. The average rank for the combined group is calculated: $\overline{R} = \frac{N+1}{2} = 5$.

6. The value of *D* is calculated by the formula:

$$D = n_1\left(\overline{R_1} - \overline{R}\right)^2 + n_2\left(\overline{R_2} - \overline{R}\right)^2 + n_3\left(\overline{R_3} - \overline{R}\right)^2, \quad (3)$$

where *D* represents an analogue of the between-group variation and depends on the sizes of the groups.

7. The Kruskal-Wallis statistic H is calculated by the formula:

$$H = \frac{12}{N(N+1)} \cdot \left[\left(\frac{\sum R^2}{n}\right) - 3 \cdot (N+1)\right], \quad (4)$$

The results of the calculations for points 5 and 6 are presented in Table 6.

8. The distribution of H approximates a chi-squared distribution with $v = k - 1$ degrees of freedom, where k is the number of groups. In our case, $k = (n-1) = 3$, so the number of degrees of freedom $v = 2$.

In our case, the critical chi-squared value $\chi^2_{crit} = 5.991$. If the value of $H > \chi^2_{crit}$, then the difference among the groups is considered statistically significant, meaning the null hypothesis is rejected. Otherwise, we accept the null

hypothesis of no differences among the groups. In our case, the value of H is greater than $\chi^2_{crit} = 5.991$, for the CFI parameters: F_0, F_m, F_v, F_v/F_m, R_{fd}.

As an example, the calculations result for points 5, 6, 7, 8, 9 are presented in Table 6.

Results of the Kruskal-Wallis Statistical Analysis:

For the CFI Parameter F_{st}, we accept the null hypothesis of no differences between groups, indicating that the test result showed no statistically significant difference between groups for this parameter at the significance 0.05. For the parameters $F_{0,}$ F_m, F_v, F_v/F_m, R_{fd} we reject the null hypothesis and accept the alternative hypothesis, indicating a statistically significant difference between groups at the significance level of 0.05.

Table 6. Results of the Kruskal-Wallis test

CFI parameters	Value of D	Kruskal-Wallis Statistic, H	Degrees of freedom k= (n-1)	χ^2 at α=0,05	Acceptance of the Null Hypothesis
F_0	48.67	6.489	2	5.991	no
F_m	54.00	7.200	2	5.991	no
F_v	54.00	7.200	2	5.991	no
F_v / F_m	54.00	7.200	2	5.991	no
F_{st}	44.67	5.956	2	5.991	yes
R_{fd}	48.67	6.489	2	5.991	no

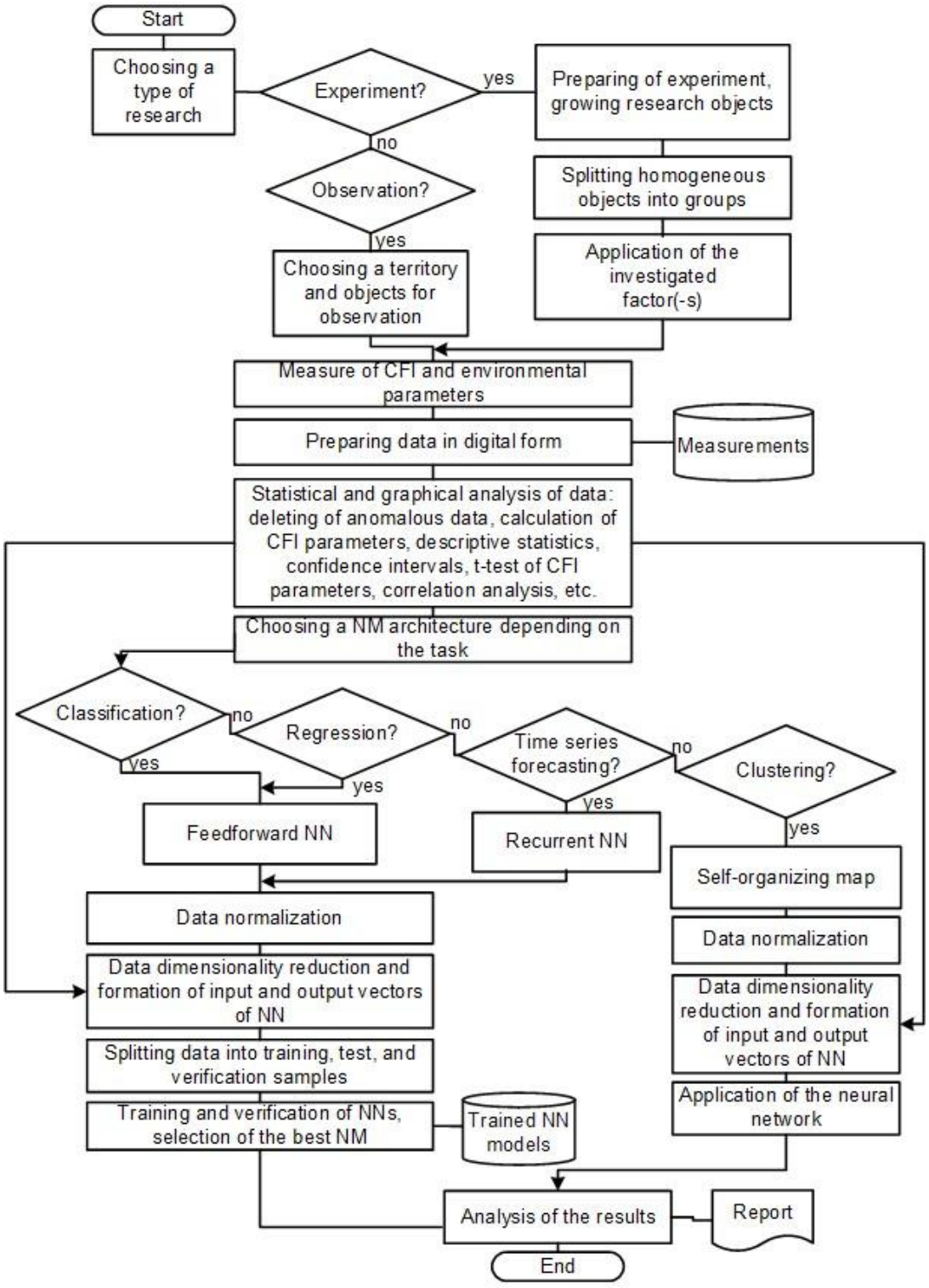

Figure 2. The generalized algorithm for studying the CFI using neural networks

Stage 7. Neural network approach

The neural network approach allows us to determine whether CFI can be used for a particular task (e.g., the ability to make a decision on the need for irrigation, to detect infections at early stages, to determine the effectiveness of plant treatment with herbicides, fertilizers, to register the impact of stress factors), as well as identify additional patterns and dependencies.

The developed generalized algorithm for studying the CFI using neural networks is shown in Fig. 2.

Conclusion

The paper presents the methodology for diagnosing the state of plants using chlorophyll fluorescence induction. It includes a list of indicator plants, signs of excessive element content in the soil, an example of an experiment scheme and an experiment log template, and describes graphical, statistical, and neural network approaches to analyzing the experiment data.

Acknowledgement

The paper is published with partial support by the ITHEA ISS (www.ithea.org) and the ADUIS (www.aduis.com.ua).

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